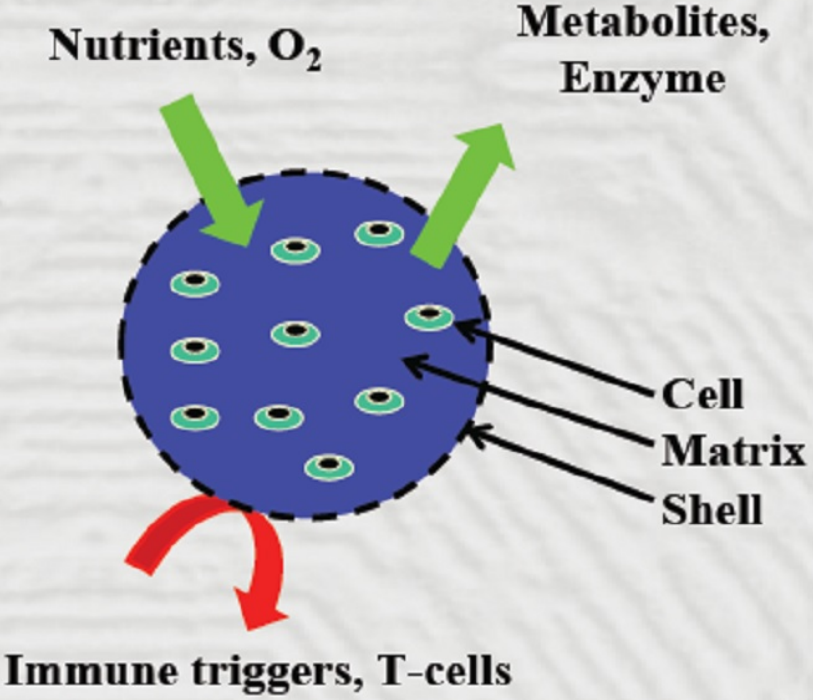
Materials for Biomedical Applications



Edited by
Mohammad A. Jafar Mazumder and Amir Al-Ahmed

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Materials for Biomedical Applications

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Special topic volume with invited peer reviewed papers only.

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Preface

Biomedical applications of materials as a form of micro to macro molecules provides an outstanding demonstration of the multi- and interdisciplinary arena of materials. The aim of this book is to provide a critical insight on scientific, engineering and processing aspects of various materials, which can ultimately contribute towards the advancement of medical sciences.

As the target audiences cover a wide interdisciplinary field, each peer-reviewed chapters written with detail background by a selected group of academic and clinical experts. This book entitled "**Materials for Biomedical Applications"** reflect the true inter-disciplinary nature of materials science, demonstrate the scientific background and interaction between the materials and biosystems, biocompatible or biodegradable polymers, materials for diagnostic, and the development of devices and enabling technologies for therapeutic applications.

This book summarises the up-to-date status of the field, covers important scientific and technological developments by many distinguished experts, who came together to contribute their research work and comprehensive, in-depth and up to date articles. Written in a versatile and contemporary style, this book can be used as an invaluable reference source for graduate students, scientist, researcher working in chemistry, polymer chemistry, polymer engineering, chemical engineering and materials science. We are thankfully appreciate the tremendous efforts and co-operation of all contributing authors for their devotion, valuable time in preparing state-of-art chapters for this book. We would also like to express our gratitude to the publishers and all authors, and others for granting us the copyright permissions to use their illustrations. Although sincere efforts were made to obtain the copyright permissions from the respective owners to include the citation with the reproduced materials, we would like to offer our sincere apologies to any copyright holder if unknowingly their right is being infringed.

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Dr. Amir Al-Ahmed, would like to take this opportunity to express his sincere thanks to Dr. Haitham M. Ba-Haidarah (Director CORE-RE, KFUPM) and also to his colleagues at the King Fahd University of Petroleum & Minerals, Saudi Arabia for their never-ending support and co-operation.

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Table of Contents

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Pentose Phosphate Pathway in Disease and Therapy

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Keywords: Pentose phosphate pathway, glucose-6-phosphate dehydrogenase, transketolase, NADPH, nucleotide biosynthesis, glutathione, metabolic diseases, non-metabolic diseases, cancer, epilepsy, Alzheimer's disease, chemotherapy, gene therapy, chemoresistance, metabolomics, metabolic flux analysis, novel drug discovery and development.

Abstract. Pentose phosphate (PP) pathway, which is ubiquitously present in all living organisms, is one of the major metabolic pathways associated with glucose metabolism. The most important functions of this pathway includes the generation of reducing equivalents in the form of NADPH for reductive biosynthesis, and production of ribose sugars for the biosynthesis of nucleotides, amino acids, and other macromolecules required by all living cells. Under normal conditions of growth, PP pathway is important for cell cycle progression, myelin formation, and the maintenance of the structure and function of brain, liver, cortex and other organs. Under diseased conditions, such as in cases of many metabolic, neurological or malignant diseases, pathological mechanisms augment due to defects in the PP pathway genes. Adoption of alternative metabolic pathways by cells that are metabolically abnormal, or malignant cells that are resistant to chemotherapeutic drugs often plays important roles in disease progression and severity. Accordingly, the PP pathway has been suggested to play critical roles in protecting cancer or abnormal cells by providing reduced environment, to protect cells from oxidative damage and generating structural components for nucleic acids biosynthesis. Novel drugs that targets one or more components of the PP pathway could potentially serve to overcome challenges associated with currently available therapeutic options for many metabolic and non-metabolic diseases. However, careful designing of drugs is critical that takes into the accounts of cell's broader genomic, proteomic and metabolic contexts under consideration, in order to avoid undesirable side-effects. In this review, we discuss the role of PP pathway under normal and abnormal physiological conditions and the potential of the PP pathway as a target for new drug development to treat metabolic and non-metabolic diseases.

Introduction

Every living organism, whether it is an unicellular prokaryote or a multicellular eukaryote, possess metabolic pathways to break-down large organic molecules into small intermediary compounds, to generate energy for cellular synthesis [1]. While thousands of enzymes, substrates and co-factors are involved in these reactions, anomalies of the metabolic pathway often affect the life-style of the most complex and multicellular organism on earth, i.e., human. Human being combats much different type of diseases throughout their lifetime. Human disease is defined by impairment of normal functioning because of disordered or abnormal conditions of an organ or the whole body, which may results from the effects of genetic or developmental errors, infection, nutritional deficiency, toxicity, or other unfavourable environmental factors [2]. The cause and effect of disease are multifaceted. Many diseases are caused by pathogenic microorganisms (e.g., virus-influenza, fever, bacteria-tuberculosis,

diarrhoea; parasites-malaria, dengue), while others are due to mutation in the genetic element (e.g., autoimmune disease-Systemic lupus erythematosus) or both (e.g., cancer). The effect of the disease can be either acute or chronic. However, the severity of the disease can be life threatening, if it remains undetected or untreated.

During the early 20th century, invention of the antimicrobial drug Penicillin, saved lives of thousands of soldiers and civilians from syphilis, staphylococcal and streptococcal infections [3]. New drugs, vaccines, diagnostics and surveillance systems against epidemic diseases like malaria, cholera, pneumococcal infection, tuberculosis, sexually transmitted diseases (STDs) and severe acute respiratory syndrome (SARS) saved thousands of lives worldwide. Insulin and chemotherapy drugs save or increase the longevity of thousands of lives that are suffering from non-infectious diseases such as diabetes or cancer. Malaria and cholera caused by parasites and bacteria, respectively, used to appear in epidemic forms in many developing countries of South-East Asia [4–8]. On the other hand, diabetes, cancer, Alzheimer's disease, etc., that are associated with genetic factors or other stimuli are now global problem as these have turned into an epidemic form in many developed countries [9-11]. Whatever the cause of the disease is, treatment of diseases certainly impose socio-economic burden for the affected countries. Unfortunately, in recent years, an additional cost has been added to the existing costs associated with the management of human diseases is drug resistance. Not only pathogenic bacteria, but even cancer cells are showing drug resistance properties, causing relapses at later stages of treatment. As a result, it has become highly important to develop new drugs or modify existing treatment strategies.

Design and development of novel drugs require knowledge on the metabolic pathways of the infected cells as well as disease causing organisms, whenever applicable [12]. Researchers already identified that many pathogens or even cancer cells adopt alternative pathways for nucleotide synthesis and cell metabolism under diseased conditions. Traditional therapies or new targeted therapies mend abnormal functions of single genes and proteins, as well as affect a narrow range of metabolic downstream reactions. However, metabolic networks inherently possess wide functional flexibility due to the presence of multiple alternate macromolecule synthetic pathways. As a result, targeted drugs may fail to control a pathologic phenotype that eventually develops drug resistance. To overcome these problems, detailed information on the metabolic pathways could pave a solution to the problem of 'drug resistance' for the drug developing companies.

Use of next generation sequencing technology and proteomics has advanced our understanding of the metabolomics under pathological conditions. Although these data are highly informative, additional information on the cell's energy state or co-factors might provide useful information under pathological conditions. In this context, metabolic flux analysis (MFA) is a robust technique to understand the biological reactions. MFA applies tracers (e.g., ^{13}C labelled glucose or acetate or ^{14}N labelled glutamine) to detect and calculate the metabolic state of cells [12-14]. In the field of metabolic engineering, this has been applied vastly for strain improvement of biotechnologically important prokaryotic or simple eukaryotic organisms to understand the effect of genetic alterations, changes of external conditions and different nutritional status. Since, mammalian cells exert heterogeneous nature under diseased condition, several tracers are applied to obtain information on diverse metabolites and these data are integrated to the transcriptomic and proteomic data to unravel the correct pathway under pathological condition. Perhaps a similar approach can be applied to narrow down metabolic pathways associated with drug resistance, as well.

This review is focussed to discuss the attributes of one of the most important metabolic pathways known as the pentose phosphate (PP) pathway, and human diseases and therapy associated with this pathway. This is the only pathway for nucleotide biosynthesis in both prokaryotes and eukaryotes. There are now ample evidences from in vitro studies that suggest that cancer cells or drug resistant organisms utilize PP pathway for abnormal proliferation or biomass accumulation [15-17]. Defects in the enzymes or genes of this pathway have already been known to be associated with inborn error, heritable or non-heritable diseases, and even with colon cancer, breast cancer etc. Therefore, PP pathway could be a target for new drug development or for modification of existing drug therapies. Therapeutic approaches to correct the mutation of a particular gene or several genes using gene

therapy or introduction of small molecule inhibitors or combinations could be promising agents to treat the drug resistance properties of cancer cells.

Overview of the Pentose Phosphate Pathway

Pentose phosphate pathway is present in every living organism. The history of the metabolic role of this pathway dates back to 1926. Patients treated with the malarial drug primaquine led to the first medical description of a drug- induced hemolytic anemia that correlated with an intrinsic defect of red blood cell metabolism. In 1948, glucose-6P-dehydrogenase, the first rate limiting enzyme of the PP pathway was discovered. In 1960, this pathway was conceived as part of glucose metabolism, based on studies on enzymatic activities and metabolites in yeast [18]. Now it is well established that once glucose enters the cell, it can be used in three major pathways: glycogen synthesis, glycolysis and the PP pathway. While glycogen is used for glucose storage, glycolysis is considered as the key metabolic pathway for glucose metabolism and requires oxygen to generate energy in the form of ATP, NADH and pyruvate. On the other hand, only a very small fraction of glucose (5-30%) is metabolised through the PP pathway. The interplay between the glycolytic pathway and the pentose phosphate pathway are highly dependent on the metabolic state and growth rate of the organism. In human being, PP pathway is most active in liver, mammary glands and adrenal cortex of the brain [19].

Branches of the PP Pathway

Cytoplasm or cytosol is the site where pentose phosphates are synthesized from hexose sugars through a series of biochemical reactions. Biochemically, the pentose phosphate pathway is divided into two branches: oxidative branch and the non-oxidative branch. The oxidative branch is operated by three enzymes: glucose 6-phosphate dehydrogenase (G6PDH), 6-phospho gluconolactonase (6PGL) and 6- phosphogluconate dehydrogenase (6PGDH). The substrate of the oxidative branch, glucose-6-phosphate (G6P), is generated from the glycolytic pathway and is oxidized into 6-phosphogluconolactone by G6PDH with the production of NADPH. The unstable lactone ring is then opened by lactonase into 6-phosphogluconic acid and undergoes oxidative decarboxylation by the 6PGDH enzyme, which irreversibly produces ribulose-5-phosphate and a second NADPH and CO_2. The resulting ribulose-5-phosphate can be further converted into ribose-5-phosphate and used for the synthesis of nucleotides or can be converted into xylulose-5-phosphate and fed into the non-oxidative branch of the pentose phosphphate pathway [20] (Fig. 1).

The non-oxidative branch of the pentose phosphate pathway is operated by 4 different enzymes synthesizing 3-7 carbon containing molecules. Unlike the oxidative branch, enzymes of the non-oxidative branch take part in reversible reactions and this occurs mainly during the inter-conversion of ribulose-5-phosphate into ribose-5-phosphate or ribulose-5-phosphate into xylulose-5-phsophate. Enzymes of the non-oxidative branch are ribose-5-phosphate isomerase (R5PI), ribose-5-phosphate epimerase (R5PE), transketolase (TK) and transaldolase (TA). R5PI and R5PE convert ribulose-5-phosphate, synthesized at the oxidative branch, into ribose-5-phosphate and xylulose-5-phosphate, respectively. Ribose-5-phosphate and xylulose-5-phosphate are then catalyzed by TKs into glyceraldehyde-3-phosphate (3- carbon) and sedoheptulose-7-phosphate (7-carbon). These two intermediates are further catalyzed by TAs into fructose-6-phosphate and erythrose-4-phosphate. Erythrose-4-phosphate then reacts with a second xylulose-5-phosphate mediated by the TKs, into the final products fructose-6-phosphate and glyceraldehyde-3-phosphate. These final products are either recycled into the glycolytic pathway for energy production or reintroduced into the PP pathway for biosynthetic reactions [20] (Fig. 1).

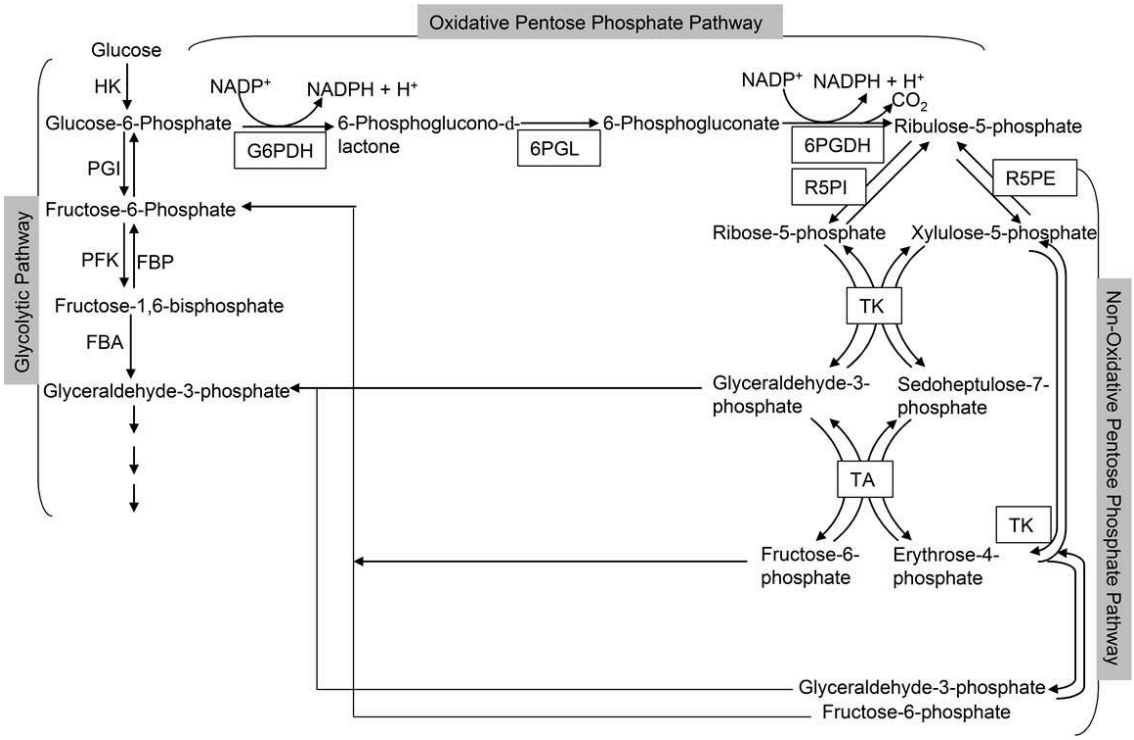

Fig. 1. Different branches of pentose phosphate pathway and their links to the glycolytic pathway. G6PDH: Glucose-phosphate dehydrogenase; 6PGL: 6-phospho glucono lactonase; 6PGDH: 6-phosphogluconate dehydrogenase; R5PI: Ribose-5-phosphate isomerase; R5PE: Ribose-5-phosphate epimerase; TK: Transketolase; TA: Transaldolase; HK: Hexokinase; PGI: Phosphoglucose isomerase; PFK: Phosphofructo kinase; FBP: Fructose bisphophatase; FBA: Fructose bisphosphate aldolase.

Functions of the PP Pathway

Metabolites synthesized in the PP pathway act as precursor molecules for macromolecule biosynthesis such as vitamin, thiamine or co-factor biosynthesis, nucleotide biosynthesis, aromatic amino acid and other amino acid biosynthesis, fatty acid metabolism or cholesterol biosynthesis, steroid hormone synthesis (Table 1). The PP pathway is also utilized for drug metabolism or detoxification reactions. In addition, NADPH synthesized through the oxidative branch, maintains a highly reducing environment to protect cells from oxidative damage. In fact, this is the main pathway for the maintenance of reducing environment during biosynthetic reactions, and for the supply of ribose sugars for the biosynthesis of nucleotides or the constituents of cellular genetic materials [16, 20-22].

Table 1. Importance of the Pentose Phosphate Pathway in cellular biosynthesis [16, 20-22].

Branches of the PP pathway	Products or intermediate compounds of PP PATHWAY	Biological Function
Oxidative branch	NADPH	Provides reducing power for biosynthetic reactions ii) Protects cells against oxidative stress, either by neutralizing reactive oxygen species (ROI) or indirectly via regenerating reduced glutathione (GSH) from its oxidized form GSSG which is previously produced by GSH peroxidase (GSHPx)-catalyzed reactions Utilized by the cytochrome P_{450} monooxygenase system to degrade xenobiotic compounds or drugs
Non-oxidative branch	Pentose-5-Phosphates: Ribose-5-P 2'deoxy ribose-5-P 5-phosphoribosyl-1-pyrophosphate (PRPP)	i) Structural components of nucleotides: a. Basal structural component of RNA b. Basal structural component of DNA c. Precursor of both de novo and 'salvage' synthesis of nucleotides ii) Intermediate products of purine metabolism and act as precursor molecules of cofactors, e.g., riboflavin, flavin mononucleotide (FMN), flavin adenine di nucleotide (FAD) iii) Precursor of the amino acid, Histidine.
	Erythrose-4-Phosphate	i) Precursor of vitamin B6. ii) Precursor of aromatic amino acid.
	Sedoheptulose-7-P	Important constituent of bacterial cell wall.
	Fructose -6-P	Recycled to the glycolytic pathway to maintain the intracellular level of Glucose-6P, which primes both the glycolytic and PP pathway and required for storage of glycogen or starch.
	Glyceraldehyde-3-P	Recycled to the glycolytic pathway for ATP synthesis.

Regulation of the PP Pathway

Metabolic flux analysis of the substrates of the two branches of PP pathway showed that the flux of the oxidative part of the PP pathway is about 14 times higher than that of the nonoxidative part in brain. Again, high ratios of oxidative versus nonoxidative part of the PP were found for the mammary gland (33:1), adipose tissue (20:1), lung (11:1), and skeletal muscle (9:1) [20-21, 23-24]. These clearly indicate that cells require significantly high reducing environment for biosynthetic reactions and for the optimum activities of cells, the two branches must be stringently regulated. Indeed, two enzymes, glucose-6-phosphate dehydrogenase (G6PDH) and transladolase (TA) act as the rate limiting enzymes for this pathway.

G-6-phosphate-dehydrogenase. *G-6-phosphate-dehydrogenase* is the first rate limiting enzyme of the PP pathway. The gene is located in the long arm of the X-chromosome at position 28. Its function was first reported in 1948 in catalyzing the first reaction in the PP pathway, providing reducing equivalents (NADPH) to all cells and defence against oxidative stress [20]. The enzyme functions as a dimer or tetramer in human cells where each monomer is composed of 515 amino

acids. G6PDH is considered as constitutively expressed housekeeping enzyme and it controls the entry of the metabolite, glucose-6-phosphate (G6P) from glycolytic pathway into the PP pathway. The activity of this enzyme is dependent on the availability of G6P and intracellular concentration of $NADP^+$ and NADPH. In addition, several genes also regulate the activity of this enzyme.

Regulation of G6PDH by NADPH Level. Unstressed cells have a higher ratio of NADPH/$NADP^+$. $NADP^+$ is required to stabilize G6PDH to its proper conformation and increases the activity of the enzyme under conditions that leads to increased NADPH formation. NADPH, on the other hand, destabilizes the enzymes conformation, lowers its stability, impairs folding and affects the kinetic parameters of the enzyme, such as concentration of G6P, $NADP^+$, pH and affinity for substrate. A variety of reactions affect the level of NADPH in cell which eventually lowers the ratio of NADPH/$NADP^+$ and modulates the activity of G6PDH. NADPH serves as the reducing equivalent for a number of reactions in the metabolic pathway. These include: glutathione (GSSG) reduction and peroxide disposal, fatty acid biosynthesis, cholesterol biosynthesis, nitric oxide (NO) production by NO synthase, superoxide production by NADPH oxidase, hydroxylation reactions, degradation of heme, polyol metabolism and the thioredoxin system [20, 23].

Glutathione (GSSG) Reduction and Peroxide Disposal. Reactive oxygen species (ROS) such as peroxides and oxygen radicals are continuously generated during aerobic metabolism in red blood cells or during oxidative fatty acid metabolism. These ROS are responsible for cellular damage such as lipid peroxidation, DNA strand breakage and protein inactivation. Glutathione (GSH) is an important component of the anti-oxidative system, being present at the mM concentration level in cell. GSH reacts directly with the ROS radicals and it is the electron donor in the reactions catalyzed by GSH peroxidises (GPx). The product of the oxidation of GSH by GPx is glutathione disulfide (GSSG). Within cells, GSH is regenerated from GSSG in the reaction catalyzed by glutathione reductase (GR), a flavoenzyme that transfers electrons from NADPH to GSSG. The cycling of GSH in the reactions catalyzed by GPx and GR depends on the availability of reduction equivalents in the form of NADPH. NADPH consumed during this process is regenerated by reactions catalyzed by G6PDH and 6-phosphogluconate dehydrogenase. Thus, clearance of peroxides from cellular system requires effective generation of NADPH and this is controlled by the GSH level [20, 23].

Fatty Acid Synthesis. In human fatty acids synthesis is highly active in liver, mammary glands and brain. Fatty acid biosynthesis occurs in the cytosol through a series of reactions where acetyl-CoA (synthesized from pyruvate, the end product of glucose metabolism) and Malonyl-CoA are linked to form palmitoyl-CoA. During this elongation process, NADPH supplies electrons to Malonyl-CoA [20, 23].

Cholesterol Biosynthesis. Cholesterol is an essential structural component of animal cell membrane, required to establish proper membrane permeability and fluidity. It is also a precursor of steroid hormones (e.g., progesterone, testosterone, estradiol and cortisol), bile acids and vitamin D. Cholesterol is synthesized from acetyl-CoA in three stages and NADPH acts as the electron donor for the first stage during which 3-hydroxy-3-methylglutaryl CoA (HMG) is converted to mevalonate [20, 23].

Nitric Oxide (NO) Production by NO Synthase. NO radicals are important signalling molecule and involved in numerous biological functions, including glutathione (GSH) synthesis (GSH), mentioned above. NO is the product of the reaction that is catalyzed by NO synthases (NOSs). Different isoforms of NOS are available in mammalian system where they are encoded by different genes. The constitutive NOS - 1 and NOS - 3 produce NO only for short periods, after activation by a raise in intracellular concentration of calcium ion (Ca^{2+}). In contrast, the inducible NOS - 2 causes a long lasting generation of high amounts of NO in the form of peroxynitrite. All isoforms of NOS need NADPH as an electron donor to produce NO and citrulline from arginine and molecular oxygen. Since NO production and expression of constitutive or inducible NOSs have been reported for neurons and different types of glial cells NADPH consumption by NOSs contributes to the regulation of the G6PDH enzyme by NO level [20, 23, 25].

Superoxide Production by NADPH Oxidase. NADPH oxidase (Nox) is a multi-subunit protein complex that uses electrons derived from NADPH to reduce molecular oxygen to superoxide. Activation of cells leads to the association of cytosolic protein subunits of Nox with the membrane-associated proteins to form the active complex that produces superoxide [20, 23].

Hydroxylation Reactions. A large number of drug-metabolizing enzymes that consist of cytochromes P450 and NADPH-dependent cytochrome P450 reductase have been reported for brain cells. The NADPH-dependent enzymes are involved in the hydroxylation of neurosteroids, the regulation of brain cholesterol homeostasis, the elimination of retinoids, and the metabolism of xenobiotics [20, 23].

Degradation of Heme. Two enzymes in heme degradation require reduction equivalents from NADPH. These are: heme oxygenase (HO) and biliverdin reductase. HO metabolizes heme to biliverdin, iron, and carbon-monoxide (CO). The latter one acts as a neurotransmitter. Biliverdin reductase, on the other hand, reduces biliverdin to bilirubin. While, bilirubin is toxic for the brain of newborns, NADPH-dependent recycling of bilirubin by biliverdin reductase plays some antioxidative role in the cells [20, 23].

Polyol Metabolism. During polyol metabolism, the enzyme aldose reductase uses NADPH as substrate to reduce glucose to sorbitol. Sorbitol is further oxidized to fructose by sorbitol dehydrogenase. Flux through the sorbitol pathway is present in the brain and bypasses the enzyme hexokinase, which is the control point of glucose metabolism. An elevated flux through the sorbitol pathway has been reported in the brains of diabetic patients. It is thought that depletion of cellular NADPH due to exaggerated flux through aldose reductase contributes to oxidative stress-mediated dysfunctions of neural cells in diabetes [20, 23].

Thioredoxin System. Thioredoxin and thioredoxin reductase are important constituent of the intracellular redox environment. Thioredoxin reductase is homologous to glutathione reductase (GR) and depends on NADPH as electron donor. The thioredoxin system can repair peroxynitrite induced disulfides in brain tubulin and therefore may be of high importance for brain cells under pathological conditions [20, 23].

G6PDH Regulation by Regulatory Genes. p53 is a transcription factor and a well known tumor suppressor gene. In cell, it plays important role in glucose metabolism and oxygen transfer. p53 directly binds with G6PDH and inhibits its function. Although the exact mechanism of p53 controlling G6PDH is not unravelled, frequent mutation of p53 and a high PP flux has been reported. Such mutations also make cells resistant to ROS. In addition to p53, TAp73, a structural homologue of the tumor suppressor gene p53 also regulates metabolism in tumor microenvironment and contributes to oncogenic cell growth. However, unlike p53, TAp73 is rarely mutated and frequently overexpressed in human tumours. TAp73 activates the expression of G6PDH and increases PP flux to direct glucose-6-phosphate to the production of NADPH and ribose-5 phosphate for the synthesis of macromolecules and detoxify reactive oxygen species (ROS) in tumor environment [26].

Non-steroidal Anti-inflammatory Drugs (NSAIDS). NSAIDs, such as ketoprofen decreases G6PDH activity. The underlying mechanisms is that, acyl-CoA, a derivative of many NSAIDs, interact with the same site that binds phosphoenol-pyruvate (PEP), a negative allosteric modulator of G6PDH. In addition, arachidonic acid, which is the precursor of prostaglandin and thromboxane (inflammatory response components) also regulate G6PDH by impairing the splicing efficiency of G6PDH pre-mRNA, via the activation of adenosine monophosphate activated protein kinase [20, 23].

Transaldolase (TA). *Transaldolase (TA)* is the rate limiting enzyme of the non-oxidative branch of the PP pathway. Human TA is located in chromosome 11 and the enzyme is encoded by a single-copy gene with 336 amino acids and a molecular weight of 38 kDa. The activity of this enzyme is dependent on the availability of its substrate ribulose-5-phosphte and inhibited by inorganic phosphate (Pi), D-arabinose-5-phosphate and glyceraldehyde-3- phosphate [20, 23]. Both, G6PDH and TA are also regulated by the glycolytic metabolites 2, 3-bisphosphate and glyceraldehyde-3-phosphates. These metabolites interconnect the PP pathway with glycolysis and gluconeogenesis and therefore, their concentration modulates the activities of G6PDH and TA [20, 23].

Diseases of the PP Pathway

Diseases associated with metabolic pathways are commonly called metabolic diseases. However, PP pathway has been associated with numerous types of diseases including cancer or malignancy. Thus PP pathway diseases can be classified into two major groups: non-malignant diseases and malignant diseases.

Non-malignant Disease. Non malignant diseases with respect to PP pathway can again be divided into two groups: metabolic diseases and non metabolic diseases.

Metabolic Diseases. Metabolic diseases are directly associated with defects on the enzymes of the particular metabolic pathways. In case of the PP pathway, defect or mutation of the oxidative branch and the non-oxidative branch enzymes have been reported, leading to abnormal accumulation or depletion of metabolites or reduced enzyme activity associated with the pathway, which is reflected in disease phenotypes.

Glucose-6-phosphate Dehydrogenase (G6PDH) Deficiency. G6PDH is present in almost all living organisms and it is expressed in all tissues and cell types from higher animals and plants to prokaryotic system. Diseases associated with reduced functioning of this enzyme are the most common heritable human enzyme defect, present in more than 400 million people worldwide [27-28]. Although most patients with the G6PDH deficiency are asymptomatic, symptomatic patients are almost exclusively male, due to the X-linked pattern of inheritance, but female carriers can be clinically affected due to unfavorable lyonization, where random inactivation of an X-chromosome in certain cells creates a population of G6PDH-deficient red blood cells coexisting with normal red cells. More than 100 missense mutations in the *G6PD* gene are known to date. The two variants (G6PDH A− and G6PDH Mediterranean) are the most commonly inherited variants. G6PD A− has an occurrence of 10% among American blacks, while G6PDH Mediterranean is prevalent in the Middle East. The known distribution of the disease is largely limited to people of Mediterranean origins (Spaniards, Italians, Greeks, Armenians, and Jews). Most of these mutations cause little or no disease. However, some mutations cause severe instability of the dimeric molecule, G6PDH. As a result, the patients suffer from lifelong chronic nonspherocytic hemolytic anemia (CNSHA). CNSHA patients are sensitive to drugs or chemicals that induces oxidative stress such as: (i) fava beans (leading to favism), (ii) antimalarial drugs, e.g. primaquine, pamaquine, and chloroquine; (iii) antimicrobial agents, e.g. sulfanilamide, sulfamethoxazole, and mafenide, (iv) thiazolesulfone, (v) methylene blue, (vi) naphthalene, (vii) certain analgesics, e.g. aspirin, phenazopyridine, and acetanilide, (viii) non-sulfa antibiotics, e.g. nalidixic acid, nitrofurantoin, isoniazid, dapsone, and furazolidone and, (ix) Henna, a herbal cosmetic has been known to cause haemolytic crisis in G6PDH-deficient infants [28].

The severity of CNSHA associated with G6PDH deficiency is that G6PDH/NADPH pathway is the only source of reduced glutathione in red blood cells (erythrocytes). Inactive G6PDH is unable to synthesize reduced form of NADPH. As a result, under oxidative stress, when all remaining reduced glutathione is consumed, enzymes and other proteins (including hemoglobin) are subsequently damaged by the oxidants, leading to electrolyte imbalance, cross-bonding and protein deposition in the red cell membranes. Damaged red cells are phagocytosed and sequestered in the spleen. The hemoglobin is metabolized to bilirubin, causing jaundice at high concentrations. In addition to jaundice, hemoglobin is excreted directly by the kidney under severe cases, causing acute renal failure [28].

Glucose-6-phosphate Dehydrogenase Over Activation. The role of G6PDH over activation has recently been implicated in the development of 'Syndrome X', also known as 'metabolic syndrome'. Metabolic syndrome is a cluster of conditions that greatly increases the risk of cardiovascular disease (CVD) and type 2 diabetes (T2D). Currently, millions of individuals around the world are affected by this disease. Patients with this disease concurrently show insulin resistance, visceral obesity or belly fat, atherogenic dyslipidemia (comprises a triad of increased blood concentrations of small, dense low-density lipoprotein-LDL, decreased high-density lipoprotein-HDL and increased triglycerides) and hypertension.

In type 2 diabetes, insulin resistance exerted by adipose, skeletal muscle and liver cells do not respond appropriately to insulin and leads to increased levels of glucose. Patients with type 2 diabetes (T2D) develop vascular diseases at the early stage which subsequently progress at an accelerated rate. Recent studies show that although T2D is an independent risk factor for CVD, both the disease share common mediators, mechanism and pathways. For example, in both ailments, glucose metabolism is severely altered and there is an up-regulation of reactive oxygen species (ROS) [29-31]. The complexity of metabolic syndromes associated with G6PDH over-activation can be explained in relation to (i) insulin resistance, (ii) obesity and (iii) dislipidemia:

(i) Insulin Resistance: Insulin is a peptide hormone, produced by beta cells of the pancreas and regulates carbohydrate/glucose and fatty acid metabolism in the body. Insulin stimulates glucose metabolism differently in different tissues. For example, in skeletal muscle and liver, insulin stimulates the synthesis of glycogen from glucose and inhibits glycogenolysis. In the liver, insulin also reduces hepatic gluconeogenesis and diminishes influx of glucose into the bloodstream. On the other hand, in adipose tissue, insulin inhibits lipolysis, stimulates glucose uptake, and regulates glucose metabolism through the PP pathway. Again, in both liver and adipocytes, insulin increases G6PDH derived NADPH that facilitates lipid metabolism and regulates several oxidoreductase enzymes. Insulin resistance has been observed when there is a decrease in the responsiveness of peripheral tissues (skeletal muscle, fat and liver) to its effects. Although the exact reason of insulin resistance is unknown, some recent studies using preadipocyte (3T3-L1 cells) showed that inhibition of G6PDH expression blocked the conversion of preadipocyte clones into the adipocyte phenotype. On the other hand, overexpression of G6PDH stimulated the expression of adipocyte marker genes, TNFα and resistin; decreased synthesis of adiponectin, elevated lipid accumulation; impaired insulin signalling; and suppressed insulin-dependent glucose uptake into adipocytes. Over-expression of G6PDH promotes the expression of pro-oxidative enzymes such as iNOS and NADPH oxidases and pro-inflammatory cytokines and that the resultant oxidative stress and pro-inflammatory signalling ultimately alters the insulin response in adipocytes [29, 30]. **(ii) Obesity:** G6PDH plays a key role in increasing fat metabolism in adipocytes and liver, induces fatty liver symptom and establishes 'Syndrome X' in humans [29,30]. **(iii) Dyslipidemia:** Features associated with dyslipidemia are high plasma TG levels, low HDL levels and high LDL levels. Insulin resistance and visceral obesity are both associated with dyslipidemia. Insulin normally suppresses lipolysis in adipocytes, but in insulin resistance, impaired insulin signalling leads to increased lipolysis, which in turn leads to increased plasma free fatty acid (FFA) levels and this results in increased level of apoB, the major component of very-low-density lipoprotein (VLDL) particles. Insulin resistance may prevent degradation of apoB by reducing the activity of lipoprotein lipase, the rate-limiting and major mediator of VLDL clearance, thereby increasing VLDL levels. In adipocytes from obese mice, G6PDH overexpression or activation impairs insulin signalling and increases both FFA and TG levels [29,30].

The symptoms of insulin resistance and increased obesity with over-expressed G6PDH required identification and characterization of the pathways for a better understanding on the cause of the metabolic syndrome. Research showed that, insulin activates two parallel pathways: the phosphoinositide-3-kinase (PI3K) pathway and the mitogen-activated protein (MAP) kinase pathway. Insulin receptor substrates (IRS) undergo tyrosine phosphorylation and then activate PI3K, leading to activation of 3-phosphoinositide dependent protein kinase 1 (PDK1) and Akt kinase. The PI3K-Akt pathway then activates the downstream proteins associated metabolic effects of insulin. For example, in skeletal muscle and adipose tissue, Akt kinase stimulates translocation of the insulin-responsive glucose transporter (GLUT4) to the cell surface, thereby increasing glucose uptake. On the other hand, in hepatocytes, PI3K-dependent signaling increases G6PDH expression and glucose metabolism through the PP pathway. Insulin resistance results in inhibition of the PI3K-Akt pathway and reduces GLUT4 translocation, thereby reducing glucose uptake into skeletal muscle and fat. On the other hand, activation of *Src* kinases stimulates hepatic G6PDH activity in hyper insulinemic and obese Zucker diabetic fatty rats. In addition, *Src* kinases and *Src* dependent phosphatases are activated in liver, fat and vascular smooth muscle in the obese and insulin-resistant *db/db* mouse and in humans. Activation of these kinases results in activation of overexpressed

G6PDH in adipocytes in obese mice and in adipocytes and stromal-vascular cells in diabetic *db/db* mice. These eventually indicate the involvement of the G6PDH and PP pathway in X-syndrome.

Insulin resistance and over-expression of G6PDH also leads to the development of vascular diseases. Over-expressed G6PDH alters ion channel function, promote cell proliferation, enhance cholesterol and fatty acid synthesis, modulate immune system function and increase oxidation, all factors involved in the development of vascular disease. And, under conditions of insulin resistance, the MAP kinase pathway continuously stimulates endothelin (ET-1) production; elevates expression of the vascular cell adhesion molecules (VCAM-1) and E-selectin, enhancing leukocyte-endothelial interactions; and stimulates growth and mitogenesis. Due to the altered glucose metabolism through the PP pathway, along with upregulated activity in the MAP kinase pathway, vascular smooth muscle cell growth and function alters substantiatively. In this manner, insulin resistance leads to vascular abnormalities that predispose to atherosclerosis [29-31].

Transaldolase (TALDO) Deficiency. TALDO deficiency was first described in 2001 with ten patients from five different families in five different geographical locations. The patients were born to consanguineous couples native to Turkey, the United Arab Emirates, Pakistan or Poland. The human TALDO gene is located on chromosome 11p15.5-p15.4, and a pseudogene is located on chromosome 1p34.1-p33. The gene consists of 5 exons. Human TALDO is a monomer of 337 amino acids and functionally active as a dimmer. The mode of inheritance is autosomal recessive and so far three homozygous mutations have been detected including: i) deletion mutations (5 patients), ii) missense mutations (in 3 patients), and iii) duplication. The deletion mutation resulted in 3 bp deletion (c.512-514delCCT) resulting in p.Ser171del. Among the missense mutations, c.574G>A (p.Arg192His) (two patients) and c.575C>T (p.Arg192Cys) (one patient) replaced arginine 192 either with histidine or cysteine. Arginine 192 is the part of the phosphate-binding site of the enzyme and required for catalytic activity. Gene harbouring the p.Ser171del mutation is transcribed, but no protein or enzyme activity is detected in fibroblasts or lymphoblasts from patient sample. Clinical features included liver disease/cirrhosis, resulting in permanent scars in tissues of all patients. Most patients showed dysmorphic features (e.g. anti-mongoloid slant, low-set ears and cutis laxa), neonatal oedema, congenital heart defects, renal problems and or intermittent hypoglycaemia [32-33].

Clinical Features Resulting From TALDO Deficiency can be Explained as Follows. TA or TALDO is the rate limiting enzyme of the non-oxidative branch of the PP pathway and G6PDH is the rate limiting enzyme of the oxidative branch of the PP pathway. The two branches of the PP pathway are interconnected to maintain the fine tuning for the synthesis of ribose-5P and NADPH production. Over-expression of TALDO in Jurkat and H9 human T cell lines resulted in decreased activity of G6PDH and 6PGDH and reduced levels of NADPH and GSH. On the other hand, reduced level of TALDO activity resulted in increased activity of G6PDH and 6PGDH and increased levels of GSH. Over expression of TALDO showed increased level of apoptosis, whereas a decreased level showed inhibition of apoptosis. However, the effect of over expression or deficiency of TALDO was cell type and species specific as opposite effect was observed in mice. In TALDO deficient patients, decreased level of $NADPH/NADP^+$ was evidenced and this eventually affected the NADPH dependent reactions during cholesterol biosynthesis, hormone metabolism and vitamin D synthesis. Decreased activity of NADPH-dependent reactions also led to haemolytic anaemia in one of the TALDO deficient patient. Since TALDO is a regulator of apoptotic signal processing, this might be related to the pathogenesis of the liver disease [32-33].

Ribose 5 Phosphate Isomerase (RPI) Deficiency. Only one patient has been reported with inborn RPI deficiency. The human RPIA gene is located at locus 2p11.2 and has 9 exons. The protein functions as homotetramer with each monomer consisting of 311 amino acids. The RPI deficient patient had two mutant alleles: i) a 1 bp deletion (c.540delG) resulting in a frameshift at codon 181 and a predicted truncated protein of 196 amino acids, and ii) a missense mutation c.182C>T, resulting in an Alanine-to-Valine substitution (p.A61V). The finding of two mutant alleles in the patient and apparently healthy parents suggested an autosomal recessive inheritance. Clinical features showed slow psychomotor development of the patient, especially delayed speech

development and a slowly progressive leuko-encephalopathy. At the age of 4 years, he developed epilepsy and from the age of 7 years, he regressed, with deterioration of vision, speech, hand coordination, walking, and seizure control. Neurological examination at the age of 14 showed leukoencehpalopathy, with severe mental retardation. Surprisingly, he did not have any problem in growth parameters, or no organomegaly or internal organ dysfunction [34].

Clinical features of RPI deficiency is described as follows: less than 5% glucose metabolism takes place by the PP in brain. Despite this minimal quantity, PP pathway plays vital role during brain development by providing ribose-5P in early development for nucleic acid synthesis and later for the turnover of RNA; and the pathway supplies NADPH for lipid and cholesterol synthesis that are important constituent for myelin, neurotransmitter production and the removal of H_2O_2 to protect cell membranes. In the RPI deficient patient, it was speculated that decreased NADPH production resulted in incomplete myelination, resulting in leukoencephalopathy. Again, the neuropathy may represent intoxication from high concentrations of polyols (ribitol and arabitol) in brain. The absence of peripheral organ involvement in the patient's organs suggests a key role for RPI in the brain [34].

NADP-linked Xylitol Dehydrogenase Deficiency. The enzyme catalyses the conversion of L-xylulose to xylitol of the non-oxidative branch of PP pathway. The symptom is known as essential pentosuria and was recognized as an inborn error of metabolism in 1892. Individuals excrete 1-4 g L-xylulose in urine per day. The disease is considered a benign condition that results from the defect in the glucuronic acid oxidation pathway [34-35].

L-arabitol Dehydrogenase Deficiency. A link to the deficiency of this enzyme was recognized in 2002 in a 16 month old girl. Clinical presentations included delayed motor development, facial dysmorphism, palatoschizis and multiple skeletal abnormalities [34-35].

Sedoheptulokinase Deficiency. Sedoheptulokinase, encoded by the gene (SHPK) catalyzes phosphorylation of sedoheptulose to sedoheptulose-7P (S7P) in human. S7P is recycled in the form of 3 and 4 carbon compounds to regulate the production of ribose and NADPH. Mutation or deficiency of this enzyme has been reported in cystinosis patients descending from European ancestry. Sequence analysis of the gene showed a large 57 kb deletion leading to the parallel deletion of the CTNS gene and the adjacent gene CARKL (now known as SHPK). While the cystinosis patients homozygous for this deletion suffer from the severe infantile nephropathic form of the disease, patients with sedoheptulokinase deficiency linked to the 57 kb deletion excrete elevated levels of sedoheptulose and erythritol in urine, and have decreased sedoheptulose phosphorylating activity. Although these symptoms reflect clinical heterogeneity in cystinosis and SHPK deficiency phenotypes, till now, an isolated SHPK deficiency has not been described [34-35].

Non-Metabolic Disease. Non-metabolic diseases are caused by mutation of genes other than the specific metabolic pathway genes or enzymes or by external stimuli that affect the activities of the enzymes of the specific metabolic pathway.

Alzheimer's Disease. Alzheimer's disease (AD) is the most common form of dementia. AD is one of the major health related concern for the developed countries. AD develops for an unknown and variable amount of time before becoming fully apparent and the disease may remain undetected for many years. Major symptoms of the disease include confusion, irritability, aggression, mood swings, trouble with language and long-term memory loss. At the adverse stage, there is loss of body function and finally leads to death. Initially the disease was thought to be age related. But recent observations showed that the disease affects differently with different individuals. Mild cognitive impairment (MCI) is considered as an increased risk factor for developing AD. Patients with progressive MCI (P-MCI) have decreased level of ribose-5-phosphate (R5P) and increased concentration of lactic acid. Lactic acid is the end product of glycolysis under anaerobic condition. Under hypoxic conditions, more glucose is metabolized via the PP pathway. Decreased level of R5P is an indication of low PP activity. At the same time, memory loss is contributed by hypoxia or oxidative stress in these patients [36].

Cystic Fibrosis. Cystic fibrosis is a mutation in the gene for the protein cystic fibrosis transmembrane regulator (CFTR). It is an autosomal recessive genetic disorder that affects most critically the lungs, and also the pancreas, liver and intestine. CF is characterized by abnormal transport of chloride and sodium ions across the epithelial membranes, such as the alveolar epithelia located in the lungs. The cytopathological effect of CF in relation to PP pathway was studied. The intracellular localization of enzyme systems of the PP pathway coincides with the sites of active ion transport in several tissues including salivary glands. Patients with CF have a lowered PP pathway activity in airway epithelial cells and this defect exacerbate the oxidative damage if the patient is infected by bacteria or other environmental stress that induces oxidative stress. Patients with CF showed higher G6PDH activity and increased level of $NADP^+$ in the red blood cells (RBC). This in turn indicated that the RBC of CF patients are under oxidative stress [37].

Epilepsy. Epilepsy is a disease of the central nervous system (CNS). Approximately, 1% of the population world-wide are suffering from this disease. There are various types of epilepsy. They are defined by a cluster of features and epileptic syndrome: seizure types, age when seizure begins, electroencephalogram (EEG) findings, brain structure, family history and prognosis. Epilepsy syndrome include: temporal lobe epilepsy, frontal lobe epilepsy, reflex epilepsy, idiopathic partial epilepsy, symptomatic generalized epilepsy, etc. Epilepsy syndrome in children include: febrile seizures, juvenile epilepsy, Lennos-Gastaut syndrome, mitochondrial disorder etc. Although clinical features of epilepsy have no known relationship with abnormalities in the PP pathway, it is a potential therapeutic target for this disease, which is discussed in the next section [38].

Alcoholism. Alcoholism or impaired nutrition severely affects the level of thiamine in brain. Thiamine deficiency strongly affects the activity of transketolase (TK) and often leads to the severe neurological disorder, Wernicke–Korsakoff syndrome (WKS). Although thiamine administration results in normalization of neurological symptoms, the TK activity does not completely recover in all regions of the brain [39].

Asbestos Toxicity. Asbestos is a set of naturally occurring silicate minerals used commercially for its physical properties such as sound absorption, average tensile strength, its resistance to fire, heat, electrical and chemical damage, and affordability. Despite its commercial importance, asbestos fibres are toxic for human health, especially when it is in the air. Asbestos fibres such as chrysotile and crocidolite, are toxic and mutagenic for a number of cell types, including epithelium, mesothelium, and macrophages. Although the cytotoxicity of these fibres are explained based on their ability to increase the production of reactive oxygen species (ROS), via the iron-catalyzed reduction of oxygen and/or the activation of NADPH oxidase, the mechanism is related to inactivation of the PP pathway. Asbestos fibres down regulate the PP pathway in dose and time dependent manner and they specifically inactivate the G6PDH enzyme by directly interacting with the protein. As a result, the ROS molecules produced by the fibres exert cytotoxicty due to the lack of reducing cofactor NADPH, which is synthesized via the catalytic activity of G6PDH of the oxidative branch of the PP pathway. Asbestos associated disease is untreatable. Disease management focuses on the prevention of inhalation of the fibres using appropriate respiratory mask or remove patients from the workplace [40-41].

Methyl Mercury Intoxication. MeHg is a teratogenic compound and is considered to be the most toxic form of mercurial compounds. Many people are exposed to toxic doses of MeHg through the consumption of contaminated fish. Pregnant women, young children and fetus are the high risk group. Due to its hydrophilic property, methyl mercury passes freely across the biological membranes such as placenta and can produce anatomical anomalies for the fetus. While the toxicity of this compound is thought to be mediated by multiple mechanisms, research showed that it exerts its teratogenic effect via PP pathway. MeHg binds to the sulfhydral group of thiamine pyrophosphate (TPP), a cofactor of the enzyme transketolase and strictly required for the function of the enzyme. MeHg also produces free radicals. TPP interacts with free radicals and hydroperoxide producing thiochrome and thiamine sulphide. These oxidized compounds cannot function as co-factor for transketolase. As a result, contamination of TPP with MeHg severely affects the function of the enzyme transketolase. Since tranketolase activity is required for ribose production for DNA,

MeHg contamination severely affects the growth of the fetus and often leads to congenital malformation and even fetal death. Nutritional supplementation of selenium, and treatment of pregnant women with thiamine, decreased neuro-developmental MeHg bio-reactivity [42].

Infectious Diseases: Hepatitis C virus upregulates the TK and TA genes in hepatocytes, increases the availability of robose-5-phosphate and activates several enzymes involved in nucleotide biosynthesis. These pathways are upregulated at the early stage of infection by hepatitis C virus and force the host's metabolic pathway of hepatocytes to facilitate its own replication [15-43].

Malignant Disease

Cancer. Cancer is a broad group of disease associated with uncontrolled cell growth and malignancy. More than 200 different types of cancer are known. The cause of cancer is miscellaneous. Several oncogenes have been studied to the initiation of tumorogenesis and cancer progression. Some of the oncogenes are metabolic pathway associated genes. Interaction of cancer with metabolism was first hypothesized in 1924 by Dr. Warburg postulating that cancer cells adopt anaerobic glycolysis and their metabolic activities are different compared to the normal cells. Later, it was demonstrated that cancer cells metabolize by anaerobic fermentation and produces lactic acid and uses PP pathway for nucleic acid biosynthesis, cell cycle progression, maintenance of reducing environment for fatty acid biosynthesis, protect the tumor cells from oxidative stress, etc [44-45]. Some of the key factors associated with tumorogenesis and PP pathway are discussed below:

PP Pathway and Tumor Cell Proliferation and Survival. Under normal conditions of growth, the activity of G6PDH is maintained at minimal level to reserve cell's energy system. However, maximum activity of G6PDH and TK enzymes coincided with increased pentose monophosphate or hexose monophosphate ratio during late G1 and S phases leading to functional progression in the cell cycle. Although G6PDH works at a basal level in non transformed cells, it can exert a strong proliferative role when it becomes deregulated and is found to be associated with high proliferation rate compared to the normal cells. It was also observed that when G6PDH is exogenously expressed, plenty of ribose-5-phosphate and NADPH is detected in transformed cells. These two metabolites strongly favor the oncogenic transformation of cells, when G6PDH is over-expressed and/or hyper-activated. Tumor cells also showed higher flux in the non-oxidative branch of the PP pathway. Reactions of the non-oxidative branch are reversible and it directs fructose-6-phosphate and glyceraldehyde-3-phosphate into the glycolytic pathway. Very high levels of fructose-6-phosphate and glyceraldehyde-3-phosphate were reported in tumor cells. All these evidences suggest involvement of the PP pathway with high proliferation rates in tumor cells [15, 45].

In many oncogenically transformed tissues, simultaneous upregulation of the glycolytic pathway and the PP pathway enzymes, such as, pyruvate kinase, lactate dehydrogenase and G6PDH has been reported, too. The non-oxidative branch enzyme TA is upregulated in tumors such as bladder, brain, breast, esophageal, ovarian and testicular cancers. The other enzyme, TK, on the other hand shows variable activity and this depends on the type of tissues. It needs to be mentioned here that in human, transketolase gene family includes TK, TKTL1 and TKTL2. In transformed tissues, there is often a reduction of native TK, accompanied by an abnormal overexpression of the TKTLs. TKTL1 and TKTL2 are often mutated in tumors. As TKTL works at a higher basal rate than TK, a high TKTL/TK ratio confers a higher flux of intermediates through the non-oxidative branch of the PP pathway and facilitates the supply of ribose-5-phosphate for dividing cells. TKTL1 expression also correlated with cell proliferation rate and tumor progression. In addition to the expression levels, genetic variants of PP enzymes involving the TKTLs and the presence of specific polymorphisms in the TA gene has been associated with an increased risk of squamous cell carcinoma of the head and neck. Increased activities of G6PDH and TK were also reported in human colon cancer cell line HT29. Their activities are induced by insulin-like growth factor-1 (IGF-1) or by the heat shock protein, Hsp27. these observations suggested the involvement of PP pathway in cancer cell progression.

PP Pathway in Tumor Cell Death. Apoptosis is a process of programmed cell death in multicellular organisms that leads to characteristic cell change and death observed by blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation. Apoptosis is controlled by a diverse range of cell signals, including the intracellular levels of ROS and GSH which indicate the cells oxidative stress level. Since the PP pathway enzyme G6PDH plays master role in regulating the reducing environment, it was observed that G6PDH plays anti-apoptotic and pro-survival role in tumor microenvironment. In support of this, human promyelocytic HL-60 cells were found to be resistant to apoptosis and also showed increased activity of the PP pathway. Again, in PC-12 neural cells, BALB/c fibroblasts, G6PDH deleted embryonic stem cells and in peripheral blood mononuclear cells of G6PDH deficient patients, H_2O_2 mediated cell death was greatly increased. On the other hand, over-expression of G6PDH significantly resisted apoptosis in these cells. Similarly, knockdown of G6PD in A375 melanoma cells were cytotoxic by the strong oxidant diamide, when compared to parental cells. In addition to the G6PDH, the homologs of TK, TKTL1 gene also play antiapoptotic role as seen in human embryonic kidney cells HEK293 cells stably transfected with TKTL1 [15, 45].

PP pathway is also up-regulated during tumor invasion stage and during angiogenesis. It was observed that in breast epithelia, the level of the PP pathway intermediates such as sedoheptulose-7-phosphate (S7P) and hexose phosphate are increased. Surprisingly, the high levels of these metabolites are detectable only at the early stage of cancer. Their level remains unchanged during the transition from non-metastatic to metastatic tumors. These indicates invasive role of the PP pathway during transformation [46]. PP pathway is also associated with blood vessel growth or angiogenesis. Tumor cells secret various growth factors (e.g. vascular-epithelial growth factor, VEGF) and VEGF directly up-regulates both the oxidative and the non-oxidative branch of the PP pathway [15, 47].

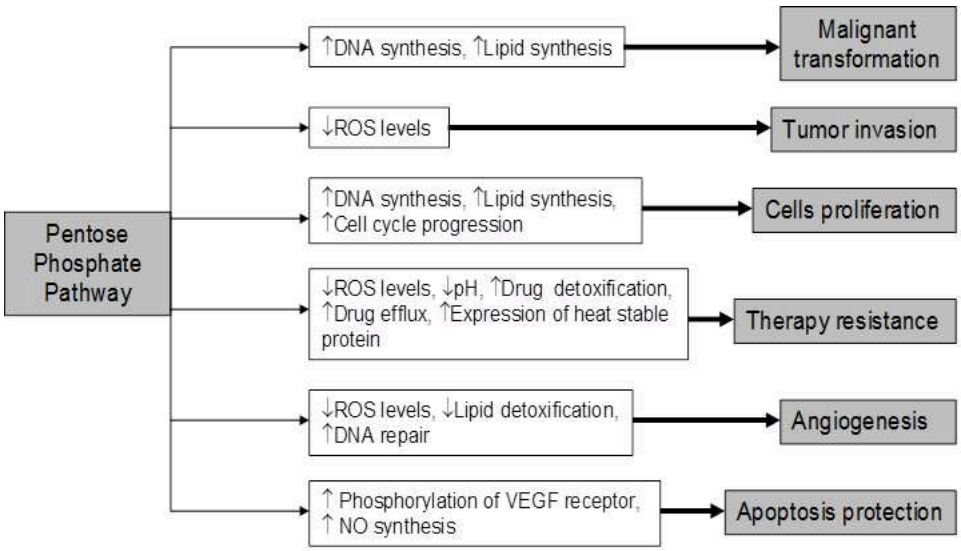

Fig. 2. Pathophysiological roles of pentose phosphate pathways in cancer.

Available Diagnostics and Treatments of PP Pathway Diseases

Treatment strategies for metabolic diseases include: i) reduction of the load of the affected pathway by substrate restriction, ii) correction of product deficiency, iii) reduction of metabolic toxicity, iv) stimulation of residual enzyme, and v) enzyme replacement [48]. Although these are some well established strategies to treat metabolic diseases, a great number of the diseases of the PP pathway are still under development.

G6PD Deficiency. *Diagnosis:* G6PD deficiency is the most common enzyme deficiency worldwide. Although an X-linked inherited disorder in which case the carrier may remain asymptomatic for life time, but affected persons show a spectrum of disease including neonatal hyperbilirubinemia, acute hemolysis, and chronic hemolysis. Therefore, the diagnosis is made by tests such as (i) quantitative spectrophotometric analysis or, more commonly, by a rapid fluorescent spot test detecting the generation of NADPH from $NADP^+$ and (ii) polymerase chain reactions. The former test result is considered positive if the blood spot fails to fluoresce under ultraviolet light. The later test is used for population screening, family studies, or prenatal diagnosis [27]. *Treatment:* The most important treatment measure is prevention – avoidance of the drugs and foods that cause hemolysis. Vaccination against some common pathogens (e.g. hepatitis A and hepatitis B) may prevent infection-induced attacks. In the acute phase of hemolysis, blood transfusions might be necessary or even dialysis in acute renal failure. Blood transfusion is an important symptomatic measure, as the transfused red cells are generally not G6PDH deficient and will live a normal lifespan in the recipient's circulation. Some patients may benefit from removal of the spleen as this is an important site of red cell destruction. Folic acid should be used in any disorder featuring a high red cell turnover [27].

Glucose-6-phosphate Dehydrogenase Over Activation. *Diagnosis:* The diagnosis of metabolic syndrome, which is suggested to be linked to G6PDH over activation is made based on several, metabolic risk factors that includes i) abdominal obesity (35 inches or more for women or 40 inches or more for men) ii) high triglyceride level (150 mg/dL or higher) iii) a low HDL cholesterol level (less than 50 mg/dL for women and less than 40 mg/dL for men) iv) high blood pressure (130/85 mmHg or higher) and v) high fasting blood sugar (100 mg/dL or higher) [49]. *Treatment:* Life style changes such as weight loss, healthy diet, physical activity and quitting smoking, with major goals of reducing the risk of heart disease and prevent the onset of type 2 diabetes, are considered as the first line of treatment for metabolic syndrome. Medicines such as statins, fibrates, or nicotinic acid to control unhealthy cholesterol levels, diuretics or ACE inhibitors to control the high blood pressure or insulin to control high blood sugar are required if life style changes are not enough. People with heart disease are also benefited by low-dose aspirin as blood thinning agent that can help reduce the risk of blood clots [49].

Transaldolase (TALDO) Deficiency. *Diagnosis:* TALDO deficiency is detected by measuring the concentrations of sugar phosphate intermediates after the addition of ribose phosphate to homogenates of erythrocytes, fibroblasts, lymphoblasts and/or liver tissue and a complete absence of TALDO activity in any patient samples. Elevated concentrations of the seven-carbon sugars sedoheptulose, mannoheptulose , sedoheptitol , perseitol and sedoheptulose-7-phosphate and the polyols erythritol, arabitol and ribitol are also detected in urine samples by liquid chromatography-tandem mass spectrometry (LC-MS). Elevations are most striking in the neonatal period compared to older age. Elevated concentrations of sedoheptulose-7-phosphate can be detected in blood spot, suggesting that newborn screening may be feasible. Prenatal diagnosis of TALDO deficiency is possible by sequence analysis of the TALDO gene in chorionic villi and amniocytes. Prenatal diagnosis is also possible by measuring sedoheptulose-7-phosphate and ribitol in amniotic fluid supernatant. In addition to measuring the intermediates and enzyme activity, sequence analysis of the *TALDO* gene is required for confirmation of the mutation [32]. *Treatment and prognosis*: Therapeutic options for TALDO deficiency are undefined. Since liver cirrhosis is common in all patients, liver transplantation is an option [32].

Ribose 5 Phosphate Isomerase (RPI) Deficiency. *Diagnosis:* The diagnosis of RPI deficiency can be made by analysis of sugars and polyols in urine, plasma, or CSF with elevated levels of ribitol, arabitol and xylulose in urine and CSF. In vitro confirmation tests include incubation of fibroblasts or lymphoblasts with either ribose-5 phosphate or 6-phospho gluconate and measure the intermediate sugar phosphates. In addition, enzyme assay and sequence analysis of the *RPIA* gene also confirms the RPI deficiency. In vivo brain MRS reveals elevated peaks of arabitol and ribitol [34]. *Treatment and prognosis:* There is no known therapeutic option for the treatment of RPI deficiency [34].

L-arabitol Dehydrogenase Deficiency. Only one patient has been reported so far. Increased level of L-arabitol was detected in urine, plasma, and CSF samples. Large amount of L- arabinose was detected in urine, too. Treatment included withdrawing fruits from the diet. This led to normalization of the polyol levels in urine samples [34-35].

Alzheimer's Disease. *Diagnosis:* Diagnosis of AD is confirmed by a brain scan and evaluation of behaviour and thinking abilities. It is the sixth leading cause of death in the United States [36, 50]. *Treatment*: Current treatments are unable to stop the disease. Except, temporarily slows the symptoms of the disease. Biomarker identification for new drug development might pave a cure for the disease. Biomarker identification of the disease has concentrated on identifying features that differentiate between those MCI subjects who will develop AD (progressive-P-MCI) from stable (S-MCI) and healthy elderly control subjects. Metabolomic studies of patient serum with an aim to identify biomarkers for disease progression or disease monitoring showed that the major contributing metabolite in the biomarker panel separating P-MCI and S-MCI patients was 2,4-dihydroxybutanoic acid. Although this is a major component of cerebrospinal fluid (CSF), it was found in plasma at nearly two orders of magnitude lower concentrations as in CSF. This metabolite is known to be overproduced under oxygen limited conditions from D-galacturonic acid, an uronic acid, which is a stereoisomer of glucoronic acid. Patients with P-MCI group showed that they have a decreased level of glucouronic acid, and metabolic pathway analysis showed that in the P-MCI group, there is a decrease in ribose-5-phosphate level and increase in lactic acid concentration. Lactic acid is the end product of glycolysis, and under hypoxic conditions, more glucose is metabolized via the PP pathway. Memory loss in AD patients is contributed by hypoxia or oxidative stress. A reduced activity of the PP pathway in these patients is in agreement with the pathogenesis of the disease [36, 50].

Cystic Fibrosis. *Diagnosis*: Individuals with cystic fibrosis can be diagnosed before birth by genetic testing or by a sweat test in early childhood [37, 51]. *Treatment*: There is no cure for cystic fibrosis. CF patients often suffer from lung infections with thick sticky mucus formation in the lungs, dehydration, diabetes, blockage in the intestines and lose of appetite. Treatment of cystic fibrosis aims to prevent the complications associated with the disease [37, 51].

Epilepsy. *Diagnosis*: Diagnosis of epilepsy is a complex, multi-step process involving patient's history on seizures, identification of seizures, thorough examination of the nervous system, and analysis of blood or other body fluids. Electroencheplaogram (EEG), CT (computerized tomography) scans, MRI (magnetic resonance imaging) scans or positron emission tomography (PET) scans is used to identify and confirm areas of the brain causing seizures [52]. *Treatment*: Treatment of epilepsy often combines anti-epileptic drugs (e.g., valproate) and changes in diet composition using high-fat, low-carbohydrate ketogenic diet [53].

Cancer. *Diagnosis*: There is no single test to diagnose cancer. Effective diagnostic tests include imaging, laboratory tests (including tests for biomarkers), tumor biopsy, endoscopic examination, surgery, or genetic testing. *Treatment*: Surgery, drugs and radiation therapy are used to treat cancer. Surgery is often the first step to treat cancer. It is used both for diagnosis and treatment of cancer. Surgery can cure many cancers. However, many cancer treatments require a combination of surgery, chemotherapy and radiotherapy. Some drug therapies used for the treatment of cancers are listed in table 2. It is noticeable, that almost all of these therapies have mild to severe level side effects or are associated with treatment failures. Again, another treatment strategy for cancer treatment is radiation therapy. Radiation therapy is used to kill or shrink tumor cells. Like drug therapies, cancer cells also show resistance to radiation therapy.

Accumulating evidence shows that G6PDH activity increases in response to oxidative stress caused by ionizing radiation to protect cells from radiation induced damage. In addition, enhanced glucose metabolism via glycolysis and PP pathway leads to the accumulation of lactic acid in tumor environment. These conditions interfere with the activity of anti-cancer drugs that are weak base (e.g., anthracyclines) and favors escapes from immune response [54]. Elevated levels of NADPH and GSH were found in many tumor cells that are resistant towards various anticancer drugs, known as multidrug resistance (MDR) [54-56].

Table 2. Existing drugs for the treatment of cancer [57-60].

Drug group	Examples	Comments
Chemotherapy	Alkylating agents: Nitrogen mustard e.g. mechlorethamine, Nitrosoureas e.g. streptozocin, Alkyl sulfonates e.g. busulfan, Triazines e.g. dacarbazine (DTIC) and Ethylenimines e.g. thiotepa	Side effects: Cytotoxicity may lead to hair loss, breathlessness, fatigue, eating challenges, memory and thinking dysfunction, nausea, neutropenia, pain etc. Recurrence Drug resistance
	Antimetabolites: 5-fluorouracil (5-FU), 6-mercaptopurine (6-MP), Capecitabine, Floxuridine, Fludarabine, Gemcitabine, Hydroxyurea, Methotrexate, Pentostatin, Thioguanine	
	Anti-tumor antibiotics: Doxorubicin, Actinomycin-D	Side effect: treatment related leukemia
	Topoisomerase inhibitors: topotecan and irinotecan (CPT-11), etoposide (VP-16), teniposide	Increased risk of secondary cancer or relapses
	Mitotic inhibtors: paclitaxel, docetaxel, ixabepilone, vinblastine	Side effect: cause peripheral nerve damage
Hormone therapy	Anti-estrogens: fulvestrant, tamoxifen Aromatase inhibitors: anastrozole, exemestane Progestins: megestrol acetate Anti-androgens: bicalutamide, flutamide Gonadotropin-releasing hormone (GnRH) agonists or analogs: leuprolide and goserelin	Side effects: Tiredness, digestive system problems, weight gain, menopausal symptoms, blood clots, effects on muscles and bones, memory problems, mood swings and depression

Immunotherapy/ Biological therapy	Monoclonal antibody therapy: rituximab, alemtuzumab Non-specific immunotherapies and adjuvants: BCG, interleukin-2 (IL-2), and interferon-α Immunomodulating drugs: thalidomide and lenalidomide Cancer vaccines: vaccine for advanced prostate cancer	Side effects: Flu-like symptoms, including fever, chills, nausea, and loss of appetite, allergic reactions, fatigue decrease in blood pressure Work better for some types of cancer than for others
Targeted therapy	Epidermal growth factor monoclonal antibodies (EGF-mABs) and small molecule receptor antagonists	Low response rate in refractory diseases
	Conjugated toxins or radioisotopes in leukemia.	Low response rate Recurrence
	Antisense oligoneucleotides	Side effects: Haemolytic anemia, renal failure, anasarca Delivery system is still unresolved Severe host response to viral vectors
	Immunoliposome encapsulated drugs	Moderate stability Inability of the carrier to leave the bloodstream or enter cells
	Small molecule inhibitors (e.g. Imatinib)	Recurrence with blast disease in chronic myeloid leukemia (CML) Drug resistance

Perspectives: PP Pathway as Target for Novel Drug Discovery

PP pathway is associated with different types of diseases in human. Many diseases discussed above showed the interaction of oxidative radicals with the metabolic pathways. Although the frequency and incidence of the metabolic diseases like RPI, TALDO etc. are not epidemic, G6PDH related diseases, cancer, Alzheimer's disease, epilepsy and metabolic syndrome are more frequent, affecting a large population worldwide. The health related tolls are also high due to the complex nature of the diseases and relapses resulting from drug resistance of the cells. Current drug approaches to treat metabolic syndrome or cancer are highly dependent on target based therapies. A major advantage of target based therapy is that they are highly effective, to treat specifically diseased cell populations, sparing the normal cells. However, their limitations reside on the dependence on a single highly modifiable target, and the rapid development of resistance based on four mechanisms: i) decrease in target protein expression ii) mutations in target genes iii) loss of target gene because of clonal selection, and iv) increased drug transport from targeted cells. To overcome these disadvantages, one solution is to re-design drugs with altered structure that will hit wider range of targets [58].

Design, development and marketing of new drug are costly procedure. However, the cumulative cost to manage the continuous emergence of drug resistance is even higher. Designing new drugs integrating a wide array of knowledge on the metabolic state of the cell may result in the discovery and development of novel drugs with higher efficacy and limited drug resistance. Although the advancement of genomics and proteomics enabled scientists to evaluate the function of a full set of genes and proteins under diseased conditions, it is also necessary to understand in full details how the genetic and protein networks interact with each other, and how they influence the metabolic network of cells to generate a particular disease phenotype. The most immediate phenotype,

describing cellular metabolism at any given instant or flux, remains an elusive target for measurement, and quantification of mammalian physiological processes in vitro and in vivo, which can provide unprecedented degree of accuracy about the diseased state. This later approach, termed metabolomics is a new field in medicine, which provides enhanced understanding of disease pathophysiology [61]. This also creates an enormous opportunity to design more effective drugs against many human diseases including cancer. Therefore to consider new drug development, metabolomics, genomics and proteomics have to be integrated [58-62] (Fig. 3).

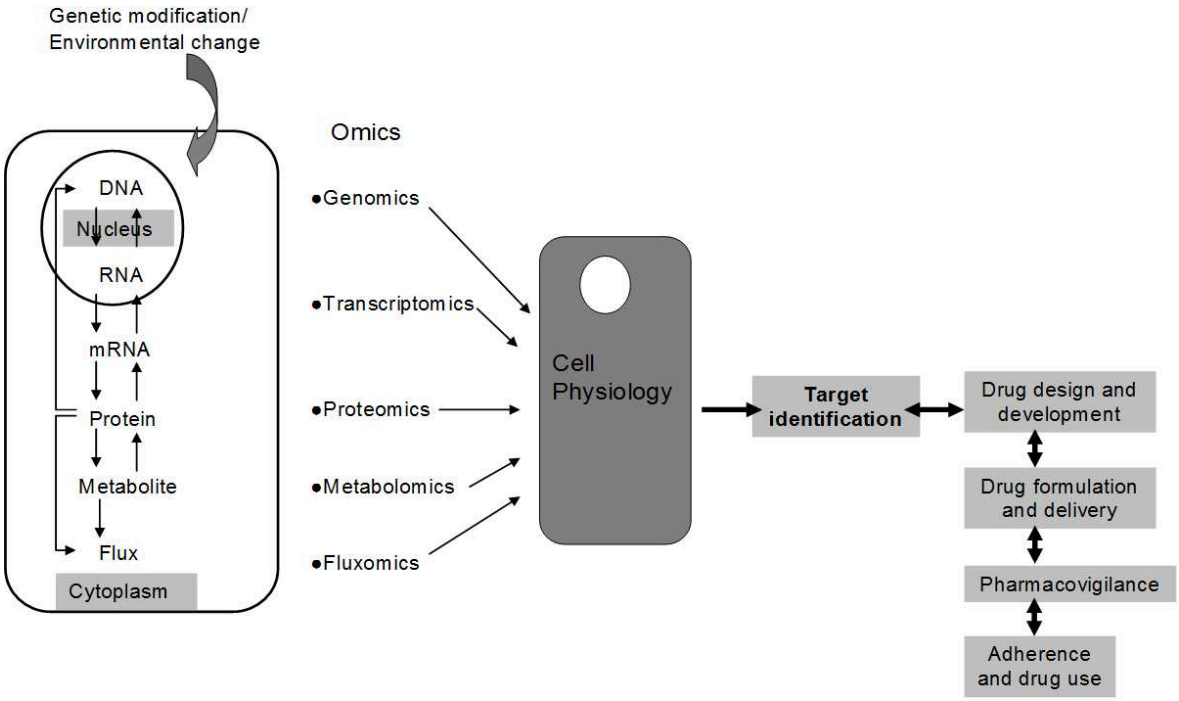

Fig. 3. Model for novel drug design and development through integration of metabolomics, genomics, transcriptomics and proteomics to understand cell's physiological conditions and identification of drug targets.

PP pathway related disease that leads to severe forms of chronic nonspherocytic hemolytic anemia (CNSHA) resulting from G6PDH deficiency, does not have definitive treatment at this moment. Since the clinical manifestations of the disease are confined to red blood cells (RBCs) and white blood cells (WBCs), all of which in turn derive from hematopoietic stem cells (HSCs), it is in principle an excellent target for gene therapy. Rovira et al (2011) developed retroviral vectors pseudotyped with the vesicular stomatitis virus G glycoprotein that harbours the human G6PDH (hG6PD) complementary DNA. Stable and lifelong expression of hG6PD was obtained in all the hematopoietic tissues of 16 primary bone marrow transplant (BMT) recipient mice and 14 secondary BMT recipients. Their findings showed the integration of a functional gene in totipotent stem cells, confirmed from enzyme activity measurement of G6PDH in peripheral blood cells of the transplanted mice. The enzyme activity was twice of that of untransplanted control mice. In addition, expression of normal human G6PDH in hG6PD-deficient primary hematopoietic cells and in human hematopoietic cells engrafted in non-obese diabetic/severe combined immunodeficient mice (SCID) was obtained. This approach of gene therapy shows promise for the treatment of severe CNSHA caused by G6PDH deficiency [63-64].

Similar to the target selection strategy described for G6PDH deficiency, targeting G6PDH ubiquitously with nonselective antagonists may not be an effective approach to the treatment of Syndrome X, which is caused by G6PDH overactivation, considering the multifaceted, beneficial effects of G6PDH on host physiology. Currently two classes of G6PDH inhibitors are known for their activity in vivo, which are i) analogs of $NADP^+$ (e.g., 6-aminonicotinamide), which block G6PD competitively and ii) 17-ketosteroids (e.g., **epi**-androsterone and dihydroepiandrosterone), that inhibit the enzyme noncompetitively. Although 6-aminonicotinamide is a highly specific G6PDH inhibitor, its side effects include neurotoxicity. On the other hand, the steroids **epi**-androsterone and dihydroepiandrosterone have reduced specificity and possess typical steroidal side-effects. However, efforts to minimize these side effects are currently underway through the development of novel inhibitors of G6PDH steroid analogs. However, the major problem with all of these drugs is their non-specific, ubiquitous activity against G6PDH. For a successful outcome in the treatment of syndrome X, it is highly important to first determine the body site, where G6PDH is overexpressed and then develop strategies for site-specific reduction of the activity of the target enzyme. For example, in case of T2D, the therapeutic goal is to reduce G6PDH activity in adipocytes, liver and vascular smooth muscle, without affecting the enzyme activity in endothelial cells or blood cells [29].

Relationship of the PP pathway in Alzheimer's disease (AD) has been studied to some extent. Accumulation of R5P mentioned in earlier sections are also associated with the low level of thiamine in the brains and peripheral tissues of patients with AD. Thiamine pyrophosphate (TPP) is the cofactor of the enzyme transketolase (TK). In AD patients, structural abnormality of TK is reported. Other proteins that link thiamine to AD pathology are apolipoprotein E (apoE), a-1-antitrypsin, pyruvate dehydrogenase complex, p53, glycogen synthetase kinase-3b, c-Fos gene, the Sp1 promoter gene, and the poly (ADP-ribosyl) polymerase-1 gene. Oral administration of thiamine showed improvement in the cognitive function of patients with AD. Unfortunately, absorption of thiamine is poor in elderly individuals. In the early stage of thiamine-deficient encephalopathy (Wernicke's encephalopathy), parental administration of thiamine has been used successfully. Further studies to determine the therapeutic value of parental thiamine is needed as a treatment for AD [65].

About one-third of patients with epilepsy have recurrent seizures with conventional treatments. Therefore, it is essential to develop new therapies against epilepsy. Imaging the CNS of children with Lennox–Gastaut and infantile spasms showed that there is decreased glucose utilization between seizures and excessive glycolysis immediately before and during seizures. In addition, a cerebral deficit in the reduced form of glutathione (GSH), which is an important scavenger of free radicals, is also reported. Since, seizures are associated with altered glucose metabolism and deficit in reduced glutathione, an inhibitor of the glycolytic pathway enzyme, phospho glucose isomerase (PGI) has been proved to be effective in the treatment of epilepsy. In addition, another intermediate, fructose-1, 6 di-phosphate (FDP) is also under investigation as this metabolite exerts potent feedback inhibition on the activity of phosphofructokinase-1, a rate limiting enzyme in glycolysis. FDP has been demonstrated as an inhibitor of seizures induced by chemical convulsants and this action is comparable to that of valproate, the conventional drug for the treatment of epilepsy. Valproate inhibits the function of GABA transaminase in the brain and also acts as an inhibitor of the enzyme histone deacetylase 1 (HDAC1), and blocks the voltage-gated sodium channels. This drug possess adverse side effect on human embryo, and in adult, it may cause fatal, fulminating impairments of hematopoietic or pancreatic function, renal impairment, weight gain, hyperammonemia and may cause brain damage. In women, there may be increased risk of polycystic ovary syndrome. In addition, the drug also causes cognitive dysfunction and Parkinsonian symptoms. The other option for the treatment of epilepsy involves combination therapy including valproate and ketogenic diet. However, ketogenic diet causes hypercholesterolemia and kidney stones in adults. To reduce the side effects of epileptic drugs, use of FDP is under investigation. FDP decreases glycolysis and preserves cellular glutathione levels, which is an important free radical scavenger in the mammalian nervous system. FDP has been safely used as a myocardial protection drug for many years, suggesting the safety of its application compared to the conventional epileptic drug, valproate. Thus strategies to

correct PP pathway abnormalities through targeting the glycolytic pathway enzymes and metabolites appeared suitable in this particular case [38].

During tumorigenesis, cancer cells exhibit mutations in tumor suppressors. Among the most mutated tumor suppressor genes in cancer is p53. It regulates many functions in the cell, including cell cycle and apoptosis. p53 also regulates the energetic status of cells through the stimulation of G6PDH of the oxidative PP pathway, β-oxidation, autophagy, and proteins that combat reactive oxygen species. In addition to p53, the AMP-activated protein kinase (AMPK) plays central regulatory role to activate glucose oxidation and decreases lipogenesis in response to unfavourable changes in AMP/ATP levels. Apparently, the AMPK-p53 pathway endow cells a metabolic adaptation to cope with energetic stresses through the regulation of ATP-producing pathways in central carbon metabolism. As these ensures important checkpoint to uncontrolled growth, loss of tumor suppressors reduces the metabolic plasticity to energetic insults or damage [58]. Consequently, MFA analyses of the pathways regulated by tumor suppressor genes or protogenes are important strategies for therapy development as this should reveal the energy level of cancer cells and with the help of genomics and proteomic data can pin point the root cause of the problem. In other words, the therapeutic approach should take an account of the energy level of cells to control cancer cell's growth and development [58, 61-62].

Mutations in proto-oncogenes enhance the metabolic potential of cancer cells in an autonomous fashion. Blanch and colleagues employed NMR-based MFA using ^{13}C glucose and showed that estrogen-stimulated breast cancer cell proliferation increases the activity of PP PATHWAY and glutamine uptake. Boros et al. used the [1,2-$^{13}C_2$] glucose tracer and showed that the transformation of A549 lung carcinoma cells by transforming growth factor b (TGF-b) diverts glucose carbons to nucleic acid synthesis via the nonoxidative reactions of PP PATHWAY. These observations show that the transformation of cancer cells involved the specific stimulation of non-oxidative PP pathway places the flux-controlling transketolase (TK) enzyme as a potential target for cancers dependent of particular transforming factors and associated kinases. A mutated isoform of TKT is upregulated in colon and urothelial tumors, suggesting that anti-TKT drugs may be effective for cancer cells exhibiting TGF-b and kinase-stimulated non-oxidative PP PATHWAY. Another set of genes that are commonly mutated in human tumors is Ras and PI3K. Using ^{13}C glucose tracers, the metabolic effects of such oncogenic signals were identified. With [1,2-$^{13}C_2$] glucose, codon-specific mutations of K-Ras differentially stimulated glycolytic activity, oxidative PP pathway and pyruvate dehydrogenase (PDH) flux, remarkably suggesting that point mutations in the same protein associated with distinct aggressiveness also communicate differently with fluxes [58].

The activation of oncogenes affects the metabolism of other nutrients, too. Up-regulation of glutamine catabolism is now recognized as an equally important metabolic switch of cancer cells. The transcription factor c-Myc (or Myc) stimulates glutamine uptake and glutaminolysis – the oxidation of glutamine to pyruvate via TCA cycle and mitochondrial $NADP^+$-malic enzyme, through the repression of the micro RNAs miR-23a and miR-23b and exerts pleiotropic effects in cell cycle and metabolism of cancer cells. Glutamine plays versatile roles in cellular metabolism: i) be oxidized for energy, ii) provide nitrogen atoms for nucleotide and hexosamine biosynthesis iii) fuel the TCA cycle with anabolic carbons, including aspartate and citrate, and iv) be partially oxidized through glutaminolysis to produce lactate and NADPH. Only a quantitative determination of the intracellular fate of glutamine can ascertain the actual impact of Myc in cancer cells. Isotopic experiments conducted by different researcher revealed glutaminolysis as a metabolic signature of glioblastoma cells that is driven by the oncogene Myc. Furthermore, Myc upregulates the transcription of ribonucleoproteins involved in the alternative splicing of pyruvate kinase (PK) [58]. These studies clearly indicates how multiple oncogenes can converge for the activation of specific pathways that enforce anabolism and at the same time reflects the involvement of several metabolic pathways in cancer progression.

It is apparent from the above discussion that PP pathway plays an important role in cancer progression. Some molecules inhibiting the PP pathway enzymes in tumor cells have been investigated (Table 3). For example, Reserveratrol induced apoptosis in colon cancer cells by

inhibiting G6PDH. Oxythiamine (OT) inhibited the activity of TK in similar type of cancer. Inhibition of either G6PDH or TK affected cell proliferation in colon cancer. Avemar, a fermented extract of wheat germ elicited apoptosis in Jurkat T cells by decreasing the activities of G6PDH and TK activity. A combination of arginine and ascorbic acid induced apoptosis in human hepatoma cell line HA22T/VGH, by inhibiting the activities of G6PDH, 6PGDH, and TA. Other inhibitors of the PP pathway are dehydroepiandrosterone (DHEA), imatinib mesylate, and genistein. Since, these inhibitors are able to inhibit the PP pathway enzymes in aggressive tumors, they can be regarded as potential therapeutic agents against invasive cancers, which usually respond poorly to conventional chemotherapy and immunotherapy.

Table 3. Summary of potential drugs that targets the PP pathway [15, 29, 38, 50, 54, 57, 59-60, 63-65].

Diseases	Potential drugs that targets the PP pathway
Non- malignant disease	
Metabolic disease	
Glucose 6PDH deficiency	Gene therapy in progress
Glucose 6PDH overexpression	6-aminonicotinamide (6-AN), **epi**-androsterone and dihydroepiandrosterone (DHEA)
L-arabitol dehydrogenase deficiency	Withdraw fruits to reduce polyol level
Non-metabolic disease	
Alzheimer's disease	Thiamine pyrophosphate
Epilepsy	Fructose-1,6-di-phosphate (FDP)
Alcoholism	Thiamine pyrophosphate
Methyl mercury intoxication	Thiamine pyrophosphate
Malignant disease: Cancer	Reserveratrol, Oxythiamine (OT), Avemar, combination of arginine and ascorbic acid, Dehydroepiandrosterone (DHEA), Imatinib mesylate, Genistein.

Conclusion

The 20th century was a ground breaking era for medical science researchers in inventing cures for many diseases that used to appear in pandemic forms, destroying the socio-economic structure of many countries of the world. Invention of antimicrobial drugs and vaccines effectively eradicated many pandemics of infectious diseases, and the development of novel chemotherapeutic drugs has shown significant progress and promises for the treatment of deadly diseases like cancer. However, the therapeutic success in these areas was also accompanied by challenges for the 21st century, particularly because of unexpected and frequent development of resistance of cancer cells to chemotherapeutic drugs. As it is discussed throughout this review, PP pathway that plays critical role in the maintenance of reducing environment and biosynthesis of nucleotides for normal cells is also linked to a wide variety of human disorders, including cancer, which is one of the most important causes of morbidity and mortality worldwide. Deregulation of PP pathway enzymes have shown to be directly involved in developing cellular phenotypes that leads to the malignant transformation of cells as well as in therapy resistance. On the other hand, therapeutic developments for many non-malignant diseases like epilepsy, Alzheimer's disease or cystic fibrosis that are linked to PP pathway are still at the level of clinical or pre-clinical trial stages. For many such drugs, effective modes of

delivery to the affected cells still remained undetermined. The design and development of these new drugs are dependent on several factors, which include concrete knowledge on the metabolic system of the host under diseased conditions. Over the past two decades, a great advancement of the high throughput techniques in the field of molecular biology and application of ^{13}C labelling experiments in the field of proteomics and metabolomics generated some data on the metabolic flux of cancer cells under diseased conditions. However, integration of these data to the drug discovery research has been limiting. While, the current focus in the treatment of human diseases is targeted therapy, based on personalized genomics, it is important to note that genes that are responsible for a particular trait do not work in an isolated manner in the highly complex and interconnected biological systems. Any change in gene expression or a mutation of a specific gene is linked to hundreds of signalling pathways and metabolic network of cells that must be considered while designing and developing new drugs. As such, a disorder of PP pathway may either be corrected through an induced change in one or more components of the PP pathway or it could even be a different factor outside of the PP pathway that could indirectly correct the error. While, the PP pathway under normal physiological conditions has been studied in great details, the precise status of this pathway under diseased status is not yet well understood. With more metabolic information being available under diseased state, it is likely that more effective treatment strategies could be formulated that can minimize undesirable outcomes in the treatment of PP pathways diseases.

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Advanced Materials for Gene Delivery

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Abstract. Gene therapy is a widespread and promising treatment of many diseases resulting from genetic disorders, infections and cancer. The feasibility of the gene therapy is mainly depends on the development of appropriate method and suitable vectors. For an efficient gene delivery, it is very important to use a carrier that is easy to produce, stable, non- oncogenic and non-immunogenic. Currently most of the vectors actually suffer from many problems. Therefore, the ideal gene therapy delivery system should be developed that can be easily used for highly efficient delivery and able to maintain long-term gene expression, and can be applicable to basic research as well as clinical settings. This article provides a brief over view on the concept and aim of gene delivery, the different gene delivery systems and use of different materials as a carrier in the area of gene therapy.

Introduction

Genes are specific sequences of bases, is the protein that carries out everyday life functions and even make up the majority of cellular structures. When genes are unable to carry out their normal functions, genetic disorders can results. Gene therapy is a technique where the disease has been treated by correcting defective genes. The process involves the introduction of a nucleic acid sequences into a cell to modify the expression of a gene in that cell. There are several approaches available that can modify the mutant or infected genes such as a) inserting normal gene into a non-specific location within the genome, and replace a non-functional gene, b) an abnormal gene could be removed from desired anatomical sources, and then swapped for a normal gene through homologous recombination, c) the defective gene could be repaired to its normal function through selective reverse mutation.

Gene therapy has attracted considerable interest to the researcher for the treatment of several diseases arising from genetic deficiencies or disorder such as cancer [1], cardiovascular disease [2], haemophilia [3], cystic fibrosis [4], β-thalassemia [5], muscular dystrophy [6], malignant tumors [7], including ovarian carcinoma [8] and HIV [9] for many years. Gene therapy has also been considered as a suitable substitute for conventional protein therapy [10]. Gene delivery for therapeutic application currently involves two strategies namely corrective or cytotoxic gene therapy. The corrective gene therapy involves the correction of genetic defects in target cells to treat a disease with single gene disorders. On the other hand, the cytotoxic gene therapy follows the destruction of target cells using a cytotoxic pathway, which can potentially be used for the treatment of uterine leimyomata and malignant tumors [4]. Despite the many research groups exploring this field, overall research progress has not lived up to the expectation in clinical trial since its first trial begins in 1970 [11, 12]. Current gene therapy is experimental, and so far the Food and Drug Administration (FDA)

in the United States has not yet approved any human gene therapy product for sale. Some of the reasons include the lack of efficient gene carriers, too rapid clearance from blood circulation, and safety concern in clinical trial. In general, all the research groups are working for the development of suitable gene therapy techniques with an objective to find an effective therapeutic gene [13] and an efficient and safe *in vivo* transfer of the genetic materials without premature degradation in systemic blood stream to the targeted tissues or organs [14].

DNA Delivery Process

The discovery of Deoxyribonucleic acid (DNA) and its function has offered us with an un-imaginable and endless possibility in various fields of science with an implication of progress of life that lead to diverse functional applications. Biotechnology is one of the many fields that has strongly benefitted from the possibilities of genetic engineering. Gene technology has facilitated the efficient production of pharmaceutical products. As a medicinal product, DNA can be delivered to patients target cells using either viral or non-viral mediated vectors. Viral vector is a virus that has been genetically altered to carry normal DNA by encapsulating and delivering their genes to target cells in a pathogenic manner. Different types of viruses are used as gene therapy vectors. On the other hand, non-viral vector method involves, the DNA molecules are usually condensed and/ or complexes with cationic transfection reagents, and then DNA constructs are engulf by the cell membrane through endocytosis and finally release the genetic materials into the target cells. Other non-viral mediated system involves creation of artificial lipids sphere with an aqueous core, which carries the therapeutic DNA, and reached to the patients target cell membrane. However, this delivery system tends to be less effective than other options.

DNA Transfection

Transfection is the delivery of DNA, Ribonucleic acid (RNA) and macromolecules into the patient target cells. A technique originally developed to allow the infected cells uptake of purified DNA rather than by intact viral or non-viral particles. In gene therapy, the DNA can be administered either by *in vivo* or *ex vivo*. *In vivo* process, the gene or vector can directly be administered into the patient or target organ, and potentially can be applied to any cell. The *ex vivo* administration includes harvesting and cultivation of cells from patients, with *in vitro* gene transfer and reintroduction of transfected cells. In pharmaceuticals or in basic research, the successes of gene therapy, either *ex vivo* or *in vivo*, mainly depends on DNA delivery to the target cells accompanied with a high level of transfection efficiency, which depends on the composition of the gene expression system. Usually the number of cells receives and express the DNA in the respective nucleus is small, and it is very difficult to improve the efficiency of DNA delivery. Moreover, correlation between the *in vitro* and *in vivo* transfection efficiency is not easy to establish [15, 16] and making conversion of positive results in cell culture into animals even more difficult. The low efficiency of DNA delivery from outside the cell to inside the nucleus is a natural consequence of this multi-step process, which involves low uptake across the plasma membrane, inadequate release of DNA molecules with limited stability, and lack of nuclear targeting. There are number of groups working using different methods for improving DNA uptake, enhancing penetration across the plasma membrane, approaches for optimizing protection and intracellular release of DNA, and ways of enhancing targeting of DNA to the nucleus.

Gene Delivery Systems

Gene therapy has the potential to improve therapeutic outcomes for currently untreatable diseases. The feasibility of widespread and well deserve conceptual gene therapy applications depends upon the development of appropriate methods for gene delivery. The main obstacle in the field of gene therapy is to find suitable vectors [17]. Currently most of the vectors actually suffer from many in directive problems such as lacking of specified cell targeting *in vivo*, inefficient long-term expression [18], and low transfection rates [19]. In addition, the vectors also grieve from achieving the optimum

packaging capacity and sufficient size of the vectors and immunogenicity. Therefore, the ideal gene therapy delivery system should have the following criterion; it should be injectable, targetable to specific sites *in vivo*, easy to be regulated, and able to maintain long-term gene expression, and should be non-immunogenic [13]. The conventional and currently engross gene delivery systems can be divided into viral and non-viral vectors with specific advantages and disadvantages [20].

Viral Vectors. Viral vectors were considered to be a potential vehicle to be explored due to its natural ability to transport their genomic DNA by encapsulating and delivering their genes to target host cells in a pathogenic manner. The process involves, the viral vector infected the patients target cells, and then viral vector off load its genetic materials (therapeutic gene) into the target cell, followed by the generation of functional cell from the therapeutic gene, and restores the target cells to a normal state. A variety of virus vectors [21, 22]such as retroviruses, lentiviruses, adenovirus, and adeno-associated virus have been extensively studied in laboratory-based research as well as in clinical trials, to deliver therapeutic genes into target living cells for displaying higher efficiencies (usually > 90%) in expression as well as delivery. Among the most studied viruses, retroviruses have been studied extensively [23]. The retrovirus entered into the cell through the interaction between receptor cell surface and the viral envelope glyco proteins [24]. Adenovirus offers an alternative way to introduce the genetic material into the target host cells. The adenovirus entered into the target host cells and produces a wide range of human infection that includes acute febrile upper respiratory infections, keratoconjunctivitis, and hemorrhagic cystitis, and then viral vector off load its genetic materials through cell lysis [25]. Considering its most attractive features, researchers made tremendous efforts to use the adenovirus in both differentiated and non-differentiated types of cells, as well as non-dividing cells and hematopoietic cells.The adenovirus is most effectively applied in cytotoxic gene therapy. The herpes simplex virus (HSV) along with thymidine kinase has also been studied in clinical trials using in situ retroviral delivery in malignant brain tumours [26] and in prostate cancer [27]. These trials have provided useful data but definitive clinical efficacy has not been marked [28]. Despite the promise of the technique, several issues like immunogenity and their oncogenic potential [29, 30], reproducibility [31], high risk in toxicity [32], cost effectiveness and non-specific uptake [33] must be resolved before consider them for long term applications.

Non-viral Vectors. As the concept of gene therapy expanded, gene therapy trigger its potential to treat currently untreatable disease that caused by genetic disorders. Viral vectors are naturally considered as an efficient vector for gene therapy, but have encountered several obstacles in clinical applications due to the issues such as high risk in toxicity, high production cost, non-specific uptake, immunogenicity, and potential gene integration into oncogenic regions. Considering these above mentioned concerns, non-viral gene delivery materials such as cationic lipids and polymers, have been extensively studied as gene delivery vectors and were found to be a promising attractive alternative to viral vectors [34]. In non-viral vector system, the genomic DNA molecules normally complexes with cationic transfection reagents, and then DNA are engulf by the cell membrane through endocytosis and release the genetic materials into the target cells. The non-viral vectors show advantageous properties in their low immunogenicity, the absence of endogenous virus recombination, low production cost and reproducibility [35]. In addition, Non-viral vectors do not depend on the size of DNA for packaging and the possibility of modification with ligands for tissue- or cell-specific targeting. Non- viral gene carriers are generally based on three categories: (i) direct DNA delivery, (ii) lipid-based and (iii) polymer-based delivery, which exist a steady driving force as gene delivery vehicles. In this review article, we will discuss the recent advances in gene delivery enlightening the focus of non-delivery vectors consisting of DNA and lipids or polymers for gene delivery.

Present Status of Non-viral Gene Delivery System

Naked DNA Delivery. Although all of the gene therapy techniques are currently under development, the simplest system involves the direct injection of naked DNA into target cells/organ, which has been under investigation for more than 40 years. The direct delivery method was useful in delivery of DNA into skeletal muscle [36], liver [37], heart muscle [38] and tumors [39]. Direct DNA delivery method has also been used in the development of the DNA tumor vaccines [40]. However, naked genes are rapidly degraded by nucleases and show poor cellular uptake [41]. Moreover, DNA and RNA are a negatively charged species and therefore don't freely passed through the negatively charged lipid cell membrane, due to electrostatic repulsion. To overcome these problems, an appropriate DNA carrier is one of the prerequisites for the success of gene therapy [42, 43]. In search for DNA carrier system that can provide safe and efficient gene delivery continues to be one of the most challenging tasks of gene therapy. The model DNA carrier should possess the following properties; a) should not be toxic or induce an immune response, and able to stabilize the DNA before and after intake, b) ability to assemble or form a stable complex or packed *in vitro*, c) must be stable enough to resist extra and intracellular enzyme (protects DNA from enzymatic degradation), d) capability of binding and passing or allowing penetration through target cell membrane by efficient nuclear targeting, e) capable to release the therapeutic DNA or RNA into the cell nuclei resulting in gene expression, f) the carrier should be mono-disperse, and less than 200 nm in diameter.

Wolff et al. [44] injected a 5% sucrose solution containing DNA into the quadriceps muscle of a number of mice and found dose-related expression that lasted for several days. However, this approach limited in its application because it can be used only with certain tissues and requires large amounts of DNA. Chang et al. used particle- mediated gene transfer (or particle bombardment) technique, which is based on coating microscopic gold particles with DNA and firing them into tissue [45]. However, it limits their long-term applications due to low level of expression and immunogeniety [46]. (Diethyl amino) ether (DEAE)-dextran [47] and calcium phosphate [48] chemicals were tried for interaction with DNA to form DEAE-dextran-DNA and Ca phosphate-DNA complex. However, both methods were hampered by cytotoxicity.

Lipid Based Gene Delivery. Lipid based gene delivery is one of the most intensively studied non-viral vectors, which appeared to be a promising technique to modulate cellular gene expression for therapeutic and research applications. They are positively charged at physiological pH, which are composed of a cationic lipid and a neutral lipid or cholesterol [49] and self-assemble with negatively charged DNA and form a positively charged complex, resulting in the formation of lipoplex [50]. Lipoplex interact with the cell membrane and internalize into the cell through endocytosis, which lead to the destabilization of the lipid complex, and results cytoplasmic delivery of the DNA. On the other hand, the use of anionic liposomes has been mainly restricted to the delivery of other therapeutic macromolecules [51].

Since 1983, researcher has been trying to develop and evaluate cationic lipids formulation for efficient gene transfer. N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) [52], [1,2-bis(oleoyloxy)-3-(trimethylammonio)propane] (DOTAP) [53], 3β[N-(N′, N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) [54], and dioctadecylamido glycylspermine (DOGS) [55] were widely used as a commercial reagent for cationic lipids. A neutral lipid, Dioleoylphosphatidylethanolamine (DOPE)is also often used in conjunction with cationic lipids to aid in endolysosomal escape [56].

N-[1-(2, 3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride, or DOTMA, was used as a lipofectin. To improve the transfection efficiencies of lipofectin, DOTMA was coupled with a neutral lipid dioleoylphosphatidylethanolamine (DOPE) in 1: 1 ratio. DOTMA was one of the first synthesized, well characterized and commercially available cationic lipids used for gene delivery. Different research groups were made their efforts to prepare modified DOTMA by varying the major functional moieties such as head group, linker, linkage bonds and hydrocarbon chains of DOTMA to reduce the toxicity and increase the transfection efficiencies [47, 57]. It was found that the

cytotoxicity associated with the formulated monovalent lipids were dependent on plated cell density and the structural aspects of the lipids [47]. {2, 3-dioleyloxy-N-[2(sperminecarboxamido) ethyl]-N, N-dimethyl-l propanaminiumtrifluoroacetate}, or DOSPA, is another cationic lipid synthesized as a derivative of DOTMA. DOSPA is structurally almost similar to DOTMA. The only difference between these two is that DOSPA has spermine group which is bound via a peptide bond to the hydrophobic chains that allows for a more efficient packing of DNA [58].

One of the best-studied cationic lipids in this setting is N- [1-(2, 3- dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTAP) [52], which has been applied to *in vivo* genetic modification of a wide variety of organs in animals [59]. The main difference between DOTAP and DOTMA is that DOTAP linked the backbone by ester bonds rather than ether bonds, which is hydrolysable and help degrading the lipids and reducing toxicity. Wang et al were able to achieve good tumor specificity using a cationic lipid system consisting of DOTAP and cholesterol. This system results in at least 10-fold greater expression per mg of tumor tissue compared to liver tissue. When the suicide gene thymidine kinase from Herpes simplex virus is delivered systemically using this system, the tumors show a marked decrease in growth [60]. 3β [N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol, or DC-Chol, was first prepared by Gao and Huang in 1991 [61]. In contrast to DOTMA and DOTAP, DC-Chol contains a tertiary amine, which could help reducing the aggregation of lipolexes leading to higher transgene expression [62].

Di-octadecyl-amido-glycyl-spermine, DOGS, is commercially available under the name Transfectam, has a structure similar to DOSPA. DOSPA and DOGS both have a spermine group and two 18- carbon alkyl chains. The difference between these two is that DOSPA has a quaternary amine. In contrast, DOGS does not have quaternary amines, chains are saturated and linked to the head group through a peptide bond. DOGS has been used to transfect many cell lines. Behr et al. showed that DOGS is very effective in delivering the chloramphenicol acetyl transferase (CAT) reporter plasmid with no noticeable cytotoxicity [55].

Polyethylene glycol (PEG), one of the readily and widely used biomaterials with very low level of toxicity, presents many attractive qualities as a liposomal coating [63]. PEG has been used to modify the surface to improve the performance of lipofection, which can facilitate the protection from degradation *in vivo*. Kim et al. showed that serum containing PEGylated lipoplexes gives increased transfection efficiencies as compared to liposomal transfection without surface attachments [64]. In addition, the PEGylated lipoplexes display improved stabilities, decreased immune responses and longer circulation times in the blood, and allow the liposome to overcome aggregation problems through mutually repulsive interactions between the PEG molecules [65]. However, the main drawback with this liposomal system is the lack of specificity with regard to cellular targeting. Shi et al. reported that PEGylation inhibited endocytosis of the lipoplexes that depends upon the functional groups that were conjugated to the lipoplexes and the amount of PEG on the liposome [66]. These awkward findings trigger the research community to design novel PEG-containing liposomes where PEG will be removed following endocytosis via degradation. Alternative liposomal formulations utilizing polymers other than PEG are being produced with the goal of creating sterically protected lipoplexes. Metselaar et al. used L-amino-acid-based polymers for lipoplex modification. It was found that L-amino-acid-polymer based lipoplex extended circulation time and reduced clearance by macrophages at similar level to those modified with PEG [63]. In addition, theses oligopeptides showed increased biodegradability and favorable pharmacokinetics when lower concentrations are used.

Anionic lipoplexes are composed of physiologically safe components that include anionic lipids, cations, and plasmid DNA [67]. In general, gene delivery by anionic lipids is not very attractive and efficient. Commonly used lipids in this category are phospholipids that can be found naturally in cellular membranes such as phosphatidic acid, phosphatidylglycerol, and phosphatidylserine. Nicolau et al [68] generated liposomes consisting of phosphatidylcholine, phosphatidylserine and cholesterol, and loaded those with a DNA designed to express insulin, followed by intravenous injection into rats, a small increase in serum insulin was observed for less than one day. These

preliminary results demonstrated the feasibility of the approach, but restricted in next step due to difficulties involve the reproducible preparation of conventional liposomes.

Encapsulation of DNA into neutral and anionic liposomes has also been explored as non-viral delivery system. An anionic liposome cannot efficiently bind negatively charged DNA due to repulsive electrostatic forces that occur between the anionic head group of the lipids and the phosphate backbone of DNA. Therefore, DNA must be encapsulated to use the anionic lipids as cell specific targeting. However, the size and shape of the DNA matrix limits it encapsulated form of applications [69].

Various anionic liposomes have been well characterized for gene delivery in a small number of cell types including CHO cells and primary hippocampal neurons [69-71]. However, despite very promising and encouraging results, overall knowledge regarding anionic lipofection is as yet limited due to many difficulties, some of which will certainly challenge our scientific ingenuity. One of the main reasons is the lack of reproducibility. Moreover, due to their systematic administration, are associated with unwanted side effects due to non- specific cytotoxicity of the immune system that leads to a variety of undesired complications. Successful gene therapy of any disease will likely be an extraordinary task with the first successes involving gene therapy effectively combined with other conventional treatments. Therefore, the relative merits of any gene delivery system have to be evaluated carefully to achieve the final target.

Polymer Based Gene Delivery. Polymer based gene delivery carriers have been developed as a replacement of viral vectors for their avoidance of immunogenic and oncogenic problems. It gained significant attention in last two decades due to their great potential for the development of safe and efficient vectors [72]. It is well understood that polymers can be specifically designed for the proposed application as synthetic polymers offer the advantage of controlled synthesis, coupling of cell or tissue specific targeting moieties, and one can easily modify the properties that confer upon specific physiological or physiochemical properties by changing the condition of synthesis like hydrophobicity, charge density and cross linking density, and molecular weight.

A large variety of synthetic and natural polymers and copolymers have been explored to find their suitability for *in vitro* and *in vivo* gene delivery applications. Unfortunately, till to date no polymeric structure has been found to be a suitable one [73]. The insight into the relationship between the polymer structure and their biological performance, such as the DNA compaction, toxicity and transfection efficiency is still rather limited. Hence, the discovery of new potent materials still relies on empirical approaches rather than on a rational design.

The natural cationic polymer that includes cyclodextrins (CDs) [74], gelatin [75], collagen [76], dextran [77, 78], cellulose [79], chitosan [80, 81], and their modified derivatives has also been researched for gene delivery applications ranging from tissue engineering to vaccination therapy [82]. Cyclodextrins (CDs) are water soluble, relatively non-toxic, and non-immunogenic cyclic oligomers of glucose units. These unique properties of cationic CDs have been utilized for several therapeutic applications [83]. Gonzalez et al. synthesized and evaluated β-cyclodextrin- based cationic polymers as potential gene carriers [84]. It was found that the transfection efficiency of β-CD polymers was comparable to that of PEI and lipofectmine. Pun et al. studied linear and branched PEIs grafted β-CD as potential gene carriers [85]. It was found that the cytotoxicity of the modified polymer was greatly reduced along with increasing the grafting percentage of CD. However, the transfection efficiency was reduced with increasing the grafting percentage of CD. Gelatin, a natural polymer derived from collagen by acid or base treatment. It contains both positive and negative charges. However, the cationic property of gelatin appears due to the lysine and arginine residues. Gelatin is biodegradable and biocompatible in physiological environment, which makes it one of the versatile materials to be used in various pharmaceutical and medical applications [86, 87]. Morimoto et al. synthesized cationic gelatins by coupling ethylenediamine (EDA) or spermine through an EDC-mediated reaction that enable interactions with biomolecules of anionic nature [88]. Xu et al. applied cationic gelatin nanoparticles for the non-viral delivery to adult canine articular chondrocytes

in vitro [89]. It was found that chondrocytes transfected with IGF-1 maintain steady IGF-1 overexpression in cationic gelatin scaffolds for up to 2 weeks in three-dimensional culture. Inada et al. investigated the transfection of cationized gelatin and plasmid DNA complex into the monocyte-derived immature dendritic cells [90]. Fujii et al. synthesized a cationic gelatin conjugate using Hemagglutinating Virus of Japan Envelope and sodium borocaptate, and studied their safety, bio-distribution and effectiveness in a multiple liver tumor model for boron neutron capture therapy [91]. Dextran, an FDA- approved highly water-soluble branched polysaccharide composed of glucose units linked by α- 1, 6- linkages. The potential application of cationic dextran as a polymeric carrier has been exploited due to its biodegradability, wide availability, ease of modification and solubility in water.A series of cationic derivative of dextran namely glycidyltrimethylammonium chloride-dextran, diethylaminoethyl-dextran and dextran-spermine have been synthesized and evaluated their transfection efficiency for the delivery of nucleic acids [92]. Dextran- spermine and their derivatives have been found to be high transfection of plasmid DNA both *in vitro* and *in vivo* [93]. Cellulose, a linear β- 1, 4- D- glucan, possesses several unique characteristics that include hydrophilicity, biodegradability and antibacterial properties have been utilized for various therapeutic applications [94]. Song et al. reported the homogeneous quaternization of cellulose in aqueous solution by preparing a novel amphiphilic quaternized cellulose derivative, hydrophilically and hydrophobically modified quaternized cellulose, and evaluated as a potential gene delivery carrier [95]. The quaternized cellulose derivatives have been found to be promising materials for gene delivery applications.

Chitosan, a biodegradable linear polysaccharide composed of D-glucosamine and N-acetyl-d-glucosamine residues with β- 1, 4- linkage. Chitosan is one of the most reported non-viral naturally-derived cationic gene polymers, due to its biodegradability, biocompatibility, non-allergenicity, mucoadhesive property and strong affinity with DNA. Chitosan demonstrated low cytotoxicity and higher transfection efficiency than poly-L-lysine (PLL) in a series of tests performed on both experimental animals [96] and humans [97]. However, the low solubility of chitosan at physiological pH limits its therapeutic applications [98]. In order to increase the solubility of the chitosan, chemically modified chitosan derivatives have been synthesized and evaluated as a carrier for asialoglycoprotein receptor-targeted gene delivery [99]. The modified chitosan interact with DNA and formed spherical self-aggregates of an average diameter of 160 nm, showed efficient transfection for COS-7 cells. Belalia et al. synthesized and investigated quaternized chitosan. The quaternized chitosan was found to be improved the mucoadhesive properties depending on the degree of quaternization, which makes this modified chitosan derivative a good candidate for gene delivery [100]. Some neutral polymers such as pluronic block copolymers [101] have also been studied. They showed good transfection efficiency. However, the mechanism between these polymers and DNA has not been identified. The problem is complicated, since the neutral polymeric vectors are only efficient *in vivo* but not *in vitro*.

Among non-viral vectors, synthetic cationic polymers have been intensively studied for gene therapy applications for self-assembling ability with DNA, controlling the size to form desired nanosized particles, protects the DNA from degradation, easy to manufacture [102], conjugate to targeting ligands and to protect the genetic material during its transport through the blood stream to the target cells [103-105]. Various synthetic cationic polymers have been used to form polyplexes with DNA, which includes polyethyleneimine (PEI) [106], poly-L-lysine (PLL) [107], poly(amino-*co*-ester) (PAE) [108, 109], and polyamidoamine dendrimer (PAMAM) [110]. Polyethyleneimines (PEIs) , first introduced by Boussif et al. in 1995 [111], in linear or branched form, is one of the most commonly studied and considered to be the gold standard of non-viral vectors for delivering genes to the target cells due to high complex stability. PEIs offer a significantly more efficient transfection and protection against nuclease degradation than other polycations such as PLL. It could be due to the fact that PEIs have higher charge density and form more compact and efficient complexation. However, PEI showed significant cellular toxicities most likely due to its high amount of positive charges [112]. In addition, due to the non-degradable nature of PEI, it is not suitable for *in-vivo* use [113]. PLL with variable molecular weights is one of the first polymers that have been studied as a

potential non-viral gene delivery carrier [114]. Poly-L-lysine, lysine as the repeating unit, is linear polypeptides; thus they possess a biodegradable nature, which is very useful for *in vivo* applications. PLL has the ability to form a polyelectrolyte complex with DNA. PLL (pKa ~10.5) is protonated in physiological conditions, may ionically interact with negatively charged phosphate groups of DNA, and form a nano-particulate polyelectrolyte complex [115]. However, *in vivo* biocompatibility of PLL/DNA complexes may be limited since the use of PLL elicited an immune response [116], and rapidly bound to plasma proteins and cleared from the circulation [117]. Poly(amino-*co*-ester) is a class of synthetic cationic polymer and hydrolytically degradable polyamines with no negative effects on cell viability were developed and explored by Langer et al. for its potential application in DNA delivery [118, 119]. The functional groups at the chain end containing amine-terminated poly(amino-*co*-ester) enhanced cellular uptake and DNA delivery [112].

Polyamidoamine dendrimers are a class of highly branched cationic polymers, which are capable of condensing DNA, and delivering it to a variety of cell lines with minimum cytotoxicity [120]. Dendrimers are spherical, highly branched polymers, most commonly used in non-viral gene delivery. The 6- generation Starburst^{TM} PAMAM dendrimers, either in intact (Polyfect®) or fractured (Superfect®) form are the most commonly used dendrimers for non-viral gene delivery. The main advantage of structural feature of these dendrimers is that they have highly dense amine in the periphery of the molecule, which enable efficient condensation of nucleic acids, leaving the inner amine functions available for a proton sponge during endolysosomal acidification, thus enabling more efficient endosomal escape. These dendrimers show significantly enhanced (>50-fold) levels of reporter gene expression compared to the intact polymer. The reason for this finding is still unclear, however it could be one of the main reasons is that an increased flexibility of the polymer with a better ability to complex DNA helps increasing the gene expression [121].

Colloidal systems with a cationic poly(lactide-*co*-glycolide) (PLGA) surface containing microparticles coated with a cationic cetyltrimethylammonium bromide surfactant by a solvent evaporation process were also reported to deliver DNA vaccine [122]. However, the microparticles attached to DNA complexes were weak as the cationic surfactant was physically adsorbed onto the microparticle surface [123].

Amphiphilic copolymers can be considered as suitable gene/drug delivery carriers [124]. Numbers of systems containing block copolymers composed of dimethylaminoethyl methacrylate (DMAEMA) and PEG [125], a random copolymer of DMAEMA and poly(ethylene glycol) monomethyl ether methacrylate (PEGMA) [126], and DMAEMA-Oligo EGMA [126] have been studied for gene delivery as PEG based polymers show a high degree of biocompatibility, and their flexible chain can easily create a favourable atmosphere, which can minimize interaction with the blood. These intriguing properties allow the PEG coated particles to circulate in the blood for an extended period of time for preferential accumulation in affected areas in the body [127]. These copolymers showed a good ability to encapsulate genes. However, they were not stable, and disintegrating into unimers especially at high salt concentrations [126], or at a lower or physiological pH [128]. The stabilization of self-aggregates could be achieved in several ways by core cross-linking such as (a) entrapment of low molecular weight monomers with the hydrophobic block followed by polymerization, which is based on the synthesis of the polymer or polymeric gel in the core to form a kind of semi-IPN core. Entanglement of the core segments to the formed gel stabilizes the particle. However, it still takes physical force to stabilize the assembly [129], (b) introduction of the polymerizable group as the side chain of the hydrophobic segment followed by cross-linking reaction [130] or polymerization in the core [131]. However, this approach limits the DNA loading capacity and also affects in drug release [132]. The stability of the micelle could also enhance through covalent cross-linking in the shell without affecting the loading capacity in the core [133]. However, the degree of incorporating functional groups into the hydrophilic shell of cross-linked micelle is low and there is no test result available with the efficiency of presenting these molecular targeting elements on the micelle. Yang et al [134, 135] developed cationic core shell micelles from an amphiphilic copolymers consisting of cholesteryl side chains in the core and a cationic amine (quaternary and tertiary amine) in the main chain as formed the shell of the micelles for co-delivery

of drug and gene. However, grafting density of the neutral lipid cholesteryl was low as they are grafted on to the backbone of the polymer chain. MW cannot be controlled as they degrade during grafting at higher temperature. Moreover, DNA binding capacity appeared to low at physiological conditions as they bind to the rigid backbone of the polymer.

Conclusion

Gene delivery is a multi-step process, in which an appropriate property of carriers would be needed to go through each step. Therefore, a major motivation for gene therapy research has been in need to rationally designed multifunctional polymeric vectors, which can overcome a series of extra- and intra-cellular barriers to develop novel treatments for diseases with no effective conventional treatment.

Viral vector is one of the important transfection vector systems, which used as an effective way of DNA delivery and efficient target cell- specific transfection. In fact, viral vector based gene therapy involves ~75% of recent clinical protocols. However, except few, no definitive success has been credited for the clinical effectiveness of viral system [136, 137]. Moreover, toxicity, restricted targeting of specific cell types, limited DNA carrying capacity, production and packaging problems, and high cost limits the application of viral medicated delivery system [138-141].

The non-viral systems, especially polymeric DNA delivery systems, have become increasingly desirable and popular research tool for elucidating gene structure, regulation, and function in both basic research and clinical applications. There are several advantages in the non-viral medicated system that includes easy and scale up production, cost effectiveness, transport, store, and easy to modulate DNA loading capacity. Moreover, the non-viral vectors can easily protect DNA from degradation in the lysosome and blood stream. The majority of non-viral vectors involve synthetic materials that offer the advantage of easy and controlled synthesis, and can easily tuned the properties of the material such as block structure, hydrophobicity, charge density, cross-linking content, and molecular weight. Additionally, synthetic polymers are available with functional groups, which can be easily modified with surface ligands to achieve targeted gene delivery via receptor- mediated endocytosis. Although, these non-viral delivery systems achieve gene transfer *in vitro* and *in vivo*, they usually show some awkward behaviour due to the size and /or the positive charge of these complexes [142]. The positively charged complexes aggregate with negatively charged blood compounds such as albumin and erythrocytes, and these large aggregates get captured in the capillary blood vessels of different organs such as lungs and liver.

The main common disadvantage of both types of vectors, which limits their *in vivo* applications, is the rapid clearance from the blood circulation to the target organs. Usually cationic polymer-DNA complexes has the tendency to form aggregated particles, which results of large size particles (~μm) with broad distribution and get captured in the capillary blood vessel, and are not stable at physiological conditions. However, for an efficient gene delivery, it is obligatory to use a carrier that is stable, mono-disperse in size, not captured in the blood vessel and can prolong its circulation time in the blood. Therefore, the relative merits of any gene delivery system have to be evaluated carefully to achieve the final target by developing an ideal system with an efficient bio-distribution to first-pass organs, rapid clearance of complexes, lack of tissue-targeting, toxicity, nonspecific interactions etc. for gene delivery that can be easily used for highly efficient delivery/ expression and can applicable to basic research as well as clinical settings.

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Weeds as Alternative Useful Medicinal Source: *Mimosa pudica* Linn. on Diabetes Mellitus and its Complications

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Abstract. Diabetes mellitus is one of the major reasons for mortality worldwide and numerous scientific studies are going on to find plausible solutions to overcome and manage diabetes and its related infirmities. Traditional medicines use medicinal plants as anti-diabetic agents and despite being a disturbing weed to farming land *Mimosa pudica* Linn. has a high traditional usage for various purposes including anti-diabetic complications. The objective of this article is to accumulate and organise literatures based on traditional claims and correlate those with current findings on the use of *M. pudica* in the management of diabetes mellitus. *M. pudica* is a creeping perennial shrub which is a common weed widely distributed in Southeast Asia specially in India, Bangladesh, Malaysia, China, Philippine etc. This plant has various species of which *M. pudica* is a well recognised plant of medicinal origin which has been traditionally used as folk medicine in India, Bangladesh and Philippine, Chinese, herbal and siddha medicines. It has wound healing, anti-diabetic, anti-diarrhoeal, antimicrobial, anti-cancer, anti-infections, anti-worm, anti-proliferative, anti-snake venom, anti-depressant and anxiolytic etc. activities. The objective of this article is to provide up-to-date information on the traditional and scientific studies based on this plant on the frontier of diabetes mellitus. The methodology followed was to methodically collect, organise and chart the recent advances in the use of *M. pudica* in diabetes and its related complications like vascular complications, diabetic wound, hyperlipidemia etc. Various scientific studies and traditional literatures clearly support the use of *M. pudica* as an anti-diabetic agent among other uses. So far, the anti-diabetic compounds have not been isolated from this plant and this can be a good scientific study for the future anti-diabetic implications.

Introduction

Diabetes mellitus is a worldwide epidemic currently being one of the top reasons of death among elder population and according to WHO projections it will be the 7th major reason of death by 2030 [1]. March 2013 update from WHO says it caused the death of 347 million people worldwide [2]. More than 80% of diabetic patients are the residents of low and middle income based countries [3]. Globally, the magnitude of diabetes is quite similar despite the sex but is seen to be slightly higher in men <60 years of age and in women at older ages [1,4].

Diabetes mellitus can be classified into four types as, (1) Type I diabetes resulting from pancreatic beta cell destruction, usually leading to absolute insulin deficiency, (2) Type II diabetes resulting from a progressive insulin secretory defect based on insulin resistance, (3) diabetes due to genetic defects in beta cell function, genetic defects in insulin action, diseases of the exocrine pancreas (such as cystic fibrosis), and drug or chemical-induced diabetes (such as in the treatment of AIDS or after organ transplantation) and (4) gestational diabetes mellitus (GDM)- diabetes diagnosed during pregnancy [1,5]. Chronic hyperglycemia can lead to long-term damage or failures to organ and metabolic inabilities. Complications that may arise are: (1) Macroangiopathy like

ischemic heart disease (IHD), stroke, peripheral vascular disease (PVD), (2) Microangiopathy like retinopathy, nephropathy, (3) Neuropathy: peripheral neuropathy, autonomic neuropathy, (4) Cataract, (5) Diabetic foot, and (6) Diabetic heart [6].

Hyperlipidemia is a related facet of diabetes mellitus since most diabetic patients have hyperlipidemia and cardiovascular complications related to it [7]. Hyperglycemia and dyslipidemia are significant and independent risk factors for the vascular complications. Dyslipidemia are said to cause pathological changes in the cardiovascular states in diabetic patients by the following molecular mechanisms like formation and accumulation of advanced glycation products, accelerated oxidative stress, protein kinase C pathway being activated, accelerated activity of hexosamine pathway, inflammation in vasculature and insulin action being impaired in the vascular tissues [7,8]. A 20% reduction of blood cholesterol level can lower approximately 31% of coronary heart disease (CHD) incidence, and 33% of its mortality rate. The known lipid lowering drugs, such as fibrates, statins and bile acid sequestrants have many side effects in patients [9].

Diabetic wound is a complication of diabetes mellitus which is characterised by the slow healing of normal wounds specially produced at the body extremities leading to diabetic foot ulceration etc. It leads to approximately 20% cases of hospitalisation, amputations, morbidity and 50% of non-traumatic lower limb amputations [10, 11]. Diabetic wounds are accelerated by the microorganisms like *Staphyloccocus aureus*, *Pseudomonas*, *Streptococci* etc. [11]. Medicinal plants have been extensively used for thousands of years to cure ailments like diabetes, blood pressure, infection etc. Herbal, Unani, Chinese, traditional or folklore all state the beneficial effects of such plants [12, 13].

Mimosa pudica Linn. is a common roadside weed which destroys crops as its roots are far reaching to the ground and are tough to remove once habituated. It is therefore not utilised to the extent fit for the abilities it possess. *M. pudica* is traditionally used in Bangladesh and India as folk-based medicine for fever, infection, anti-diabetic agent and asthma among others. In China and Philippine it is used for quite similar ailments also [14, 15]. The key focus of this article is to establish the utilitarian side of *M. pudica* and turn that into drug for future anti diabetic drug implications.

M. pudica belongs to the family Fabaceae with the synonyms like Chui mui, Najuk, Lajawanti, sensitive plant, touch-shy plant, touch me not, Varakranta, Vashini, Lajak, Lajjabati, Semalu. It is a creeping, diffusely spreading semi-woody herb or sub-shrub, with branched stems growing up to 1 m long with numerous, sparingly prickly deflexed, bristle type hairs. The leaves and pinnae are very sensitive and folds under touch. It possesses 2 or 4 pinnae arranged at the end of each petiole which are 4- 9 cm long. The leaflets are narrowly oblong, non-equilateral, 1- 1.5 cm long, sessile, sparingly bristly, with acute tips. Heads are around 1 cm in diameter and long peduncled bunched singly or about 2- 3 in each axil. Pods are flat, slightly curved, and 1-2 cm long, with 3- 5 one-sided joints that fall away when reaches maturity. Its flowers are small florets of pinkish lavender [16, 17] (Fig. 1).

Fig. 1. *Mimosa pudica* foliage and flower-heads

M. pudica grows abundantly in Bangladesh, India, Philippine, China, Malaysia and other South Asian countries and is also found in Africa and North America. Flowering time is the March-June/July. Three of *Mimosa* species namely *M. pudica*, *M. hamata* and *M. himalayana* are found quite together and have to be carefully separated. It is grown widely in open humid areas like waste lands, open grasslands usually at low and medium altitudes [15-16, 18].

Traditional Uses

It is used in Bangladesh, India, Thailand, Phillipine as an anti-diabetic agent [14, 18, 41]. According to Indian Siddha medicine, for diabetes, the juice of whole plant with roots is prescribed in 25-30 ml daily to be taken in the early morning, also the dry powder of the leaves and roots are prescribed at 2-5 g daily till the situation comes under control [18]. For itching and infection of the skin, one part of the plant juice is boiled with 1/4 of ginger oil and is applied externally over the affected area. In case of diabetic ulcers, skin infections and other ulcers the whole plant decoction is used to wash the area [14, 19, 41]. It is noteworthy to note here that the activity of *M. pudica* against bacteria and fungus were studied scientifically and was proven to be effective, and the traditional claim don't specify the type of infections although based on the inferences from scientific literature it can be deduced that the weed can effectively work against both bacteria and fungi [28, 30, 31].

Traditional use of *M. pudica* in Ecuador includes using the leaves to stuff pillows to induce sleeping in children [18, 41]. It is used in India as an anti-diabetic agent and also to get relief from boils by plastering warmed root paste of *M. pudica* [18]. In case of deep cuts and wounds the paste of roots fried in castor oil is used to prevent bleeding and healing and when fried in ghee it is good to fight tooth ache [20]. In case of furuncle, abscess and pus-filled boils the warmed paste is applied to release the pus. The leaf paste is applied on the burst boils and itches for quick healing. The leaf paste is applied on forehead to get relief from headache and migraine [20, 21, 41]. The leaf paste with honey is prescribed twice a day in empty stomach for 3-4 days for stomach ache and intestinal worms [18]. In Philippine and China it is used as anti-diabetic, wound healing, infections, burns, fistula, hypertension, as anti-depressant and anxiolytic agent, as well as for glandular swelling etc. [13, 15].

In contemporary medicine, *M. pudica* is being investigated for its potential to yield novel chemotherapeutic compounds. It contains an alkaloid called mimosine [21], which has been found to have potent anti-proliferative and apoptotic properties [22]. The pharmacological activities found based on literatures, researches and traditional uses are concisely tabulated in Table 1.

Table 1. Pharmacological activity of *Mimosa pudica* Linn.

Parts and its extract type	Pharmacological activities
Aqueous and alcoholic extracts of dried roots.	Fibrinolytic activity, Myotoxicity and toxic enzymes of *Najakaouthia* (King cobra) venom [23-25], Analgesic activity [17]
Ethanolic and petroleum ether extracts of leaves as well as herbal formulation	Antidiabetic activity [26-27], Antimicrobial activity [28]
Chloroform leaves extract	Hypolipidemic activity [29]
Methanolic and aqueous extracts of stems and roots	Anti-fungal and Wound healing activity [30-31]
Aqueous extract of stem bark/ methanolic extract of leaves and seeds	Antimicrobial activity [32-34]
Phenolic extract, methanolic extract of roots	Antiasthmatic [35], Hepatotoprotective [36]
Paste of leaves, ethanolic extract of seeds	Anticancer [37]
Aqueous leaves extract	Antimicrobial activity [30], Antimycotic activity [30]
Roots	Constipating, febrifuge, conditions of pitta, leucoderma, metropathy, ulcers, dysentery, burning sensation, vaginopathy [16], antivenom [23], Vaginal complications [38], Asthma and small pox [34], Antispasmodic [37], Emetic [39], Fever [30], Inflammations [40], Haemorrhoids, jaundice [41]

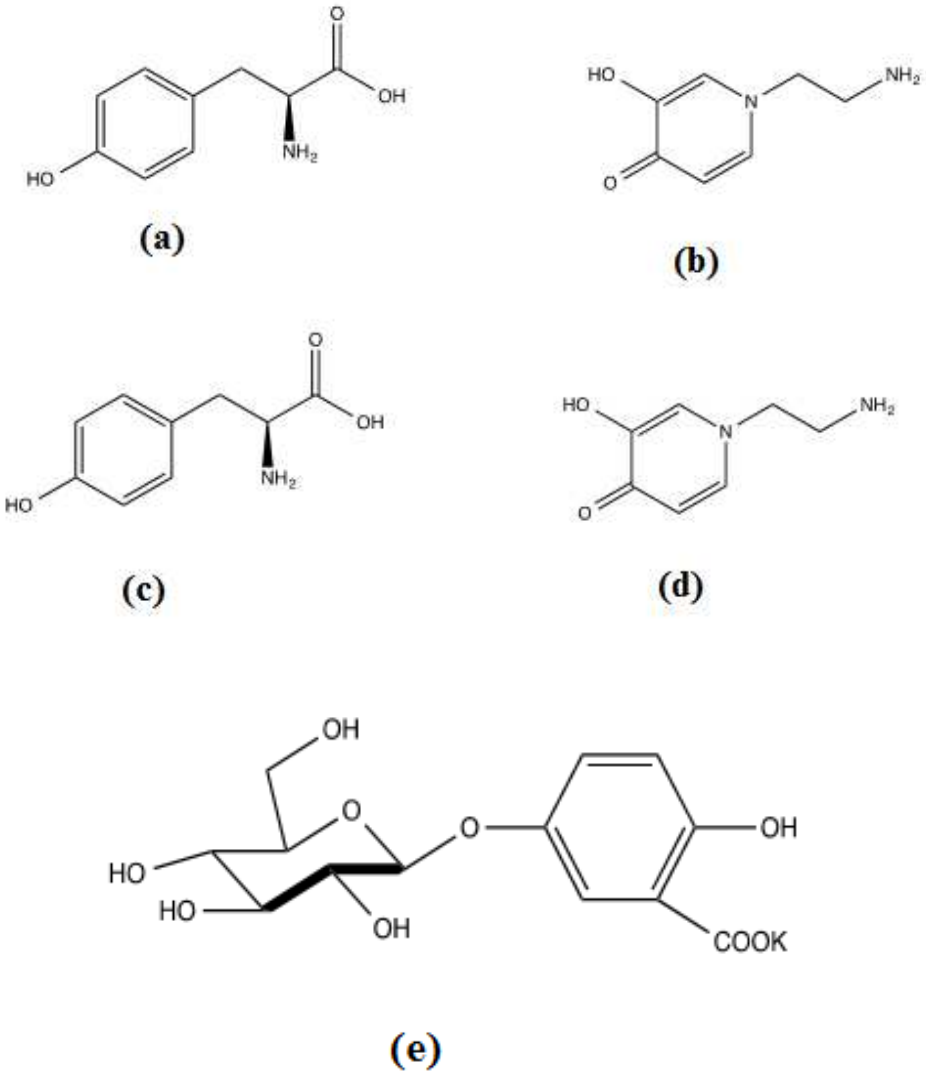
Chemical Constituents

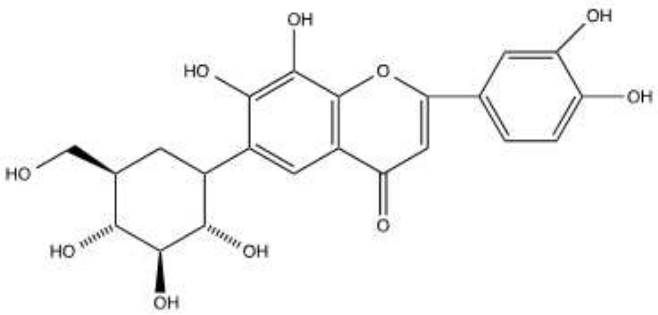
M. pudica has been reported to contain alkaloids, terpenoids, crocetin dimethyl ester, phytosterol, glycosides, flavonoids, phenolic compounds and tannins [42-43]. Gandhiraja et al [42] have reported for the presence of alkaloids, glycosides, terpenoids, flavonoids, quinone, phenol, tannins, saponins, and coumarins in the methanol extract and the same extract was devoid of steroid, anthroquinone and sugar. A recent study conducted by Tamilarasi and Ananthi [43] with methanol extract of the plant showed positive claims for the presence of alkaloids, glycosides, carbohydrates, proteins, steroids, flavonoids, and phenols but was found to be negative for tannins, quinone, saponins and terpenoids (Table 2).

Table 2. Chemical constituents from roots and leaves

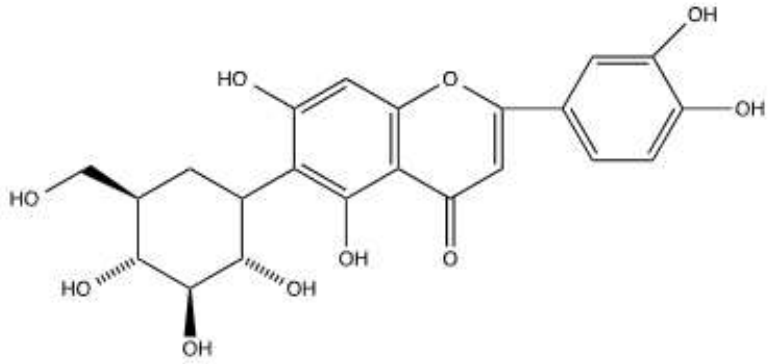
Chemical constituents from *M. pudica*		**References**
Roots	Flavonoids, phytosterol, alkaloids, amino acids, tannins, glycoside, and fatty acids	[45]
Leaves	Mimosine, terpenoids, flavonoids, glycosides, alkaloids, quinone, phenols, tannins, saponins, and coumarins	[42-43, 46]
Fresh tissues	nor-epinephrine, d-pinitol (3-mono-methyl ether of inositol) and β-sitosterol	[46]

Yuan et al. [13, 44] underwent a chemical qualitative assay on the whole plant of *M. pudica* from Hainan province of China and isolated several flavonoid glycosides. The constituents were separated and purified by column chromatography with macroporous adsorption resin Diaion HP-20, Sephadex LH-20, Toyopear HW-40, MCI Gel CHP-20, RP-18 and normal phase silica gel. Their structures were confirmed and elucidated by physical, chemical and spectroscopic analysis including IR, UV, MS, 1D and 2D NMR spectra. The isolated flavonoid glycosides were confirmed as 7,8,3',4'-tetrahydroxyl-6-C-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranosyl flavone; 5,7,4'-trihydroxyl-8-C-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranosyl flavone, 5,7,3',4'-tetrahydroxyl-6-C-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranosyl flavone, 6,7,3′,4′-tetrahydroxyl-8-C-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranosyl flavone and 5,7,3′,4′-tetrahydroxy-8-C-[β-D-apiose-(1→4)]-β-D-glycopyranosyl flavone. Zhang et al. [51] also underwent a chemical qualitative assay on the whole plant of *M. pudica* and reported five different flavonoids and their structures were determined by ^{1}H-NMR, ^{13}C-NMR and MS, and the purities of all the isolated flavonoids were calibrated by normalization method (purities > 98%). The isolated flavonoids were confirmed as 5,7,3′,4′-tetrahydroxy-6-C-[β-D-apiose-(1→4)]-β-D-glycopyranosyl flavone, isorientin, orientin, isovitexin and vitexin. Fig. 2 shows the structures of some major chemical constituents isolated from the whole plant of *M. pudica*.





(f)



(g)

Fig. 2. Chemical structures of some major chemical constituents of *Mimosa pudica*. a) Mimosine, b) Mimosinic acid, c) Tyrosine, d) Mimosinamine, e) Potassium-5-O-*β*-O-glucopyranosylgentisate, f) 7,8,3',4'-tetrahydroxyl-6-C-β-D-glucopyranosyl flavone, g) 5,7,3',4'-tetrahydroxyl-6-C-β-D-glucopyranosyl flavone.

Extensive profiling needs to be done for *M. pudica* which will elucidate the compounds showing particular activities like anti-diabetic activity. Various literatures clearly support anti-diabetic and diabetes related complications along with various others stated above. The use of major bioactives from *M. pudica* on the lowering of diabetic markers was found to be exploitable based on the literatures reviewed below in course of this discussion. The plant was found to be able to work as anti-diabetic, hypolipidemic, wound healer, anti-oxidant and free radical scavenger among other uses of this plant (see Table 3).

Table 3. Findings of *M. pudica* in diabetes and related complications.

Complications studied	Researcher/ studied by	Year
Diabetes mellitus	Umamaheswari & Prince [27]	2007
	Sutar et al. [26]	2009
	Manosroi et al. [47]	2011
Hypolipidemic agent	Rajendran & Krishnakuman [29]	2010
	Sowmya & Ananthi [48]	2011
Anti-oxidative agent	Mathew et al. [49]	2008
	Nazeema & Brindha [50]	2009
	Zhang et al. [51]	2011
	Jennifer et al. [56]	2011
Wound healing agent	Kanan et al. [31]	2009
	Paul et al. [52]	2010
	Venketeshwarlu et al. [53]	2011
	Karwani et al. [54]	2011

As Antidiabetic Agent

M. pudica is traditionally used in diabetes and is well supported by various studies [14, 18, 21, 27, 41]. Initial evaluation of *M. pudica* on diabetes was based on the evaluation of its anti-diabetic effect based on traditional formulation *Ilogen-Excel*, an Ayurvedic herbal formulation was studied by Umamaheswari & Prince [27]. *Ilogen- Excel* is composed of eight medicinal plants (*Curcuma longa, Strychnos potatorum, Salacia oblonga, Tinospora cordifolia, Vetivelia zizanioides, Coscinium fenestratum, Andrographis paniculata and Mimosa pudica*. The study was based on streptozotocin (STZ) induced diabetic rats which were administered with *Ilogen-Excel* (50 mg/kg and 100 mg/kg) for 60 days resulted in significant low blood glucose levels and significant increase in plasma insulin, hepatic glycogen and total hemoglobin. *Ilogen-Excel* was also found to decrease the levels of glycosylated hemoglobin, plasma thiobarbituric acid reactive substances, hydroperoxides, and ceruloplasmin in diabetic rats. Plasma reduced glutathione and vitamin C were seen to be significantly elevated [27].

A comparative study based on ethanolic and petroleum ether extracts of *M. pudica* were performed by Sutar et al. [26] as compared with metformin as standard drug on alloxan induced Wistar rats. The ethanolic extract showed significant decrease in blood glucose level as compared to standard drug Metformin [26]. A Thai study based on Lanna people in the Northern part of Thailand investigated five medicinal plants for the antidiabetic effects [47]. The plants were Anogeissus acuminata (Roxb. ex DC.), Gills.& Perr. (Combretaceae), Catunaregam tormentosa (Bl. ex DC.) Tirveng (Rubiaceae**)**, Dioecrescis erythroclada (Kurz) Tirveng. (Rubiaceae), Mimosa pudica Linn.var. hispida Bren. (Fabaceae), and Rauwolfia serpentina (L).Benth.ex Kurz. (Apocyanaceae). The study positively correlated *Mimosa pudica* as an anti-diabetic agent.

As Hypolipidemic Agent

A reduction of 1% cholesterol produces a 2% to 3% reduction in the risk for development of coronary heart disease [55]. *M. pudica* has lipid lowering capability which is a boon for hyperlipidemic diabetic patients. In a study for the evaluation of hypolipidemic activity of chloroform extract of *M. pudica* leaves on the reduction in lipid profiles on experimental rats was conducted against the standard drug atorvastatin by Rajendran and Krishnakuman [29]. This study performed histopathological examinations which revealed the prognosis of the disease and its effect on the organs like liver, kidney and aorta. Study ensured that *M. pudica* possess hypolipidemic properties [29]. A similar later study of hypolipidemic activity of the whole plant powder in ethanol extract was tested on dyslipidemia induced rats (high cholesterol diet) and reduction in the serum cholesterol, TG, VLDL, LDL and the concurrent increase in HDL levels as compared to standard drug lovastatine. This study showed that indeed *M. pudica* has good lipid lowering activity [48]. *M. pudica* was effective as hypolipidemic agent due to the presence of sterols which reduce cholesterol significantly by inducing faecal excretion of steroids.

As an Anti-Oxidative Agent

Reactive oxygen species (ROS) is believed to be responsible for pathogenesis of various diseases affecting tissues and liver became the basis for the scientific study of finding the anti-oxidant levels of medicinal plants for future inputs. Various diseases that evolve are found to be due to oxidative stress on cell and organs can be prevented or treated with anti-oxidants. *M. pudica* in that case is a good prospect. Mathew et al. [49] studied anti-oxidative evaluation of an aqueous extract of *M. pudica* using *in-vitro* assays on scavenging activity of anions like superoxide, hydroxyl radical, lipid peroxidation by Fe^{2+}/ascorbate system as well as Fe^{3+}/ascorbate/ADP system and nitric oxide radical. The IC_{50} (concentrations needed for 50% inhibition) of these free radicals were found to be 26.3, 156.2, 106.5, 122.1 and 101.2 µg/ml respectively. *In-vivo* evaluation of *M. pudica* by the same researchers also found that it reduced serum glucose level in streptozotocin induced diabetic rats

significantly ($p<0.005$) at a dose level of 150 mg/kg. b.w. from the 2nd hour. Among other agendas the study established that *M. pudica* was effective to control body weight, decreased serum glucose level, acted as hepatoprotective and renal functions were also seen to have improved [49].
In another similar study, the extent of lipid peroxidation (LPO) and ROS elimination along with its defense mechanisms by the enzymatic and non-enzymatic antioxidants in liver and serum was investigated [50]. Hepatotoxicity in rats (induced by ethanol) was taken as the oxidative model and efficacy of *M. pudica* was evaluated by studying the significant decrease ($P< 0.05$) in the activities of the enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, and the non-enzymatic antioxidants such as glutathione and vitamin-C. Simultaneous administration of leaves extract along with the toxin, ethanol, in rats showed a considerable protection against the toxin-induced oxidative stress and liver damage as evidence that *M. pudica* has strong antioxidant activity ($P< 0.05$). The study revealed that co-administration of the *M. pudica* aqueous extract significantly lowered the level of lipid peroxidation in alcohol-fed mice [50].

A similar Chinese study determined the total flavonoid (TF) and total phenolic (TP) contents of the ethanol extracts of the whole plant, stems, leaves, and seeds of *M. pudica* [51]. The antioxidant activity (radical-scavenging activity) of the extracts and isolated five flavonoid monomers of *M. pudica* were also evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing/antioxidant power (FRAP) assays. Along with the anti-oxidant assay the researchers also performed correlation analysis and the results showed that leaves extract possessed the highest amount of TF and TP content was significantly higher than other parts of the plant. The sequence of antioxidant activity of the ethanol extracts was found to be in descending order starting with leaves, whole plant, seeds, and stems. The sequence of effectiveness of the five flavonoid monomers were found as follows: 5,7,3′,4′-tetrahydroxy-6-C-[β-D-apiose-(1→4)]-β-D-glycopyranosyl flavone, isorientin, orientin, isovitexin and vitexin. The antioxidant activity of the first compound was equivalent to or slightly stronger than the synthetic antioxidant trolox [51].
A concurrent study done by Jennifer et al. [56] suggested that *M. pudica* could be a potential rich source of natural antioxidants. The reducing power of *M. pudica* ethanolic extract was found to be very potent and the reducing power was found to be concentration dependent. The ethanolic extract was also evaluated for HPTLC finger print at a wavelength 366 nm which showed blue and yellow coloured fluorescent zones in the tracks and were found to be flavonoids. Henceforth, it can be deduced that *M. pudica* has an abundant source of anti-oxidants apart from other bioactives which can be the reason for its broad activity against diabetes mellitus, healing of infections & wounds, hyperlipidemia etc. which are predominantly occurring due to oxidative stress.

As Wound Healing Agent

According to folklore remedy the roots of *M. pudica* have been used extensively for various types of wounds as well as diabetic ulcers [19]. This claim was studied by a handful of researchers of which an initial study by Kanan et al. [31] investigated the various solvent extracts from shoots and roots of *M. pudica* for anti-fungal wound healing activity as compared to standard gentamycin. Results showed that methanolic *M. pudica* shoot and root extracts showed the best result amongst others while the chloroform extract of root was negative for similar activity [31]. A later study done by Paul et al. [52] evaluated the chloroform and ethanolic extracts of *M. pudica* roots. The study used excision, incision, burn and dead space rat wound models using *Aloe vera* as standard wound healing agent. Both the topical and oral formulations were tried using chloroform and ethanolic extracts. The formulations were prepared in carbopol as an ointment at 2.5% & 5% concentrations and were applied topically to the wounds and meanwhile for oral preparation doses of 500 and 100 mg/kg body weight in the form of suspension. The observations found from excision and burn wound models demonstrated that both chloroform and ethanolic extracts were significant for both the doses and reduced the wound contraction and epithelisation period compared to control. The results suggested that both chloroform and ethanolic extract of *M. pudica* are well effective as oral and topical agent [52]. Later studies by Venketeshwarlu et al. [53] and Karwani et al. [54] studied wound

healing prospect of *M. pudica.* Studies showed significant anti-infection and wound healing activity for *M. pudica* due to its effect against both bacteria and fungus [28, 31, 33]. Since diabetic wounds are predominantly bacterial in nature [11] and the studies here shows it has strong wound healing activity therefore *M. pudica* can be strongly recommended for future uses and studies for diabetic wound healing activity.

Conclusion

M. pudica is abundantly grown around alongside road, land, waste area without man's help has a lot of potential since it is easily obtainable and is immensely usable for various ailments. *M. pudica* has high potential to be a good alternative to current medications based on further research and study on the active principles and various formulations. The scope of *M. pudica* lies in its ability to show good activity in so many ailments especially diabetes mellitus and its related complications all found from traditional claims and supported by scientific studies. Weed like *M. pudica* henceforth can be proposed as alternative to current medicinal plants as well as drugs and therefore provides additional medicinal sources which are cheap, handy and effective. The literatures reviewed so forth have well established that the threat of diabetes is very much now a household disease like common cold so are the medications prescribed, which are failing in curing the disease and calling on some other ones. Focusing on this problem, the weed could be proposed to be effective in treating diabetes mellitus and its related complications befitting its abilities and claims.

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Substituted Quinoline Derivatives as Potent Biological Agents

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Keywords: Quinoline derivatives, quinolone derivatives, antimicrobial, anticancer, antimalarial, antituberculosis agents.

Abstract. Quinoline derivatives are the most promising class of active pharmaceutical agents compared with other heterocyclic compounds. Biological activity profile of quinoline can easily be controlled by introducing some active pharmacophore to the core ring. This chapter deals with the synthesis of quinoline derivatives and its biological activity. The enhancement of biological activity by incorporation of active functional group and effect of these functional groups were explained briefly. Main focus is given to the synthesis of different substituted quinoline derivatives for biological application with respect to the position on the quinoline core ring and modification of quinoline ring.

Introduction

Quinoline is an important class of heterocyclic compounds found in many synthetic and natural products with a wide range of pharmacological activities. Quinolines are broad-spectrum antibiotic agents (effective for both gram negative and gram positive bacteria). The majority of quinoline antibiotics in clinical use belong to the second generation class of fluoroquinolones. Which are core quinoline framework and have a fluorine atom attached to the central ring system, typically at the C-6 position or in C-7 position. During the past couple of decades, significant numbers of quinoline based antibiotic drugs have been launched. At present, many drugs carrying quinoline are being used as effective antibiotics in the market. In spite, lots of research activities are still continuing on the synthesis of new quinoline derivatives as potential biological agents. The biological history of quinolones began in the late 1950s. The breakthrough in the drug design for the scaffold and the basic side chains have allowed improvements to be made to the first new quinolone, Norfloxacin (NFLX). The success of first generation quinolones encouraged more research in this area. Koga and his collaborators [1] introduced Norfloxacin into clinical use in 1980. They enhanced antimicrobial activity of quinolones by introducing piperidine at C-7 and fluorine atom at C-6 of basic quinolone structure, Norfloxacin. The basic piperazine ring, which can form the zwitter ion with the carboxylic acid at the C-3 position, has subsequently been shown to increase the ability of the drugs to penetrate the bacterial cells resulting in enhanced activity.

This chapter deals with the synthesis of different quinoline derivatives and its biological activity. Brief history of quinoline drugs, synthesis with respect to active functional group at different position of quinoline core are also cover.

History of Quinoline Derivative

Quinoline was first extracted from coal tar in 1834 by Friedlieb Ferdinand Runge. It is a hygroscopic liquid with a strong odour. Quinoline can be synthesised by different named reactions like, Skraup, Knorr, Combes, Friedlander, and Gould-Jacobs reaction. Quinoline nucleus is an important class of heterocyclic compounds found in many synthetic and natural products with a wide range of pharmacological activities. 4-Methanolquinoline derivatives such as Cinchona alkaloids and Mefloquine that are rapidly acting blood schizontocides, these compounds were introduced for routine use in 1985 as an antimalarial. The 4-aminoquinolines such as Chloroquine and Amodiaquine are rapidly acting blood schizontocides with some gametocytocidal activity. The 8-aminoquinolines such as Primaquine are used as tissue schizontocides to prevent relapses of the ovale and vivax malarias. In recent studies it has been established that many quinoline derivatives are being intensively used in medicinal field as anticancer [2], anti-inflammatory [3], antibacterial, antifungal [4] and antiviral [5] agents. The quinoline derivatives are also used as good antimicrobial agents. It is easily penetrate the bacterial cell wall and inhibit DNA gyrase. The antibacterial activity of quinolones depends not only on the quinolone core scaffold but also on the active pharmaphoric substituents around the ring. These substituents exert their influence on bacterial activity by providing additional affinity for bacterial enzymes, enhancing cell penetration or altering the pharmacokinetics. The antibacterial activity generated by fluoroquinolones is caused by the inhibition of two bacterial enzymes: DNA gyrase (atopoisomerase enzyme in bacteria) and topoisomerase IV enzyme. The general function of topoisomerases is to facilitate the uncoiling of DNA during DNA replication.

Chemistry of Quinoline

Quinoline is a heterocyclic aromatic organic compound. It has the formula C_9H_7N (Fig. 1) and is a colourless hygroscopic liquid. Quinoline is mainly used as a building block to bioactive molecules. Its principal use is as a precursor to 8-hydroxyquinoline, which is a versatile chelating agent and precursor to pesticides. Its 2- and 4-methyl derivatives are precursors to cyanine dyes. Oxidation of quinoline affords quinolinic acid (pyridine-2,3-dicarboxylic acid), a precursor to the herbicide sold under the name Assert. Owing to high water solubility, quinoline has significant potential for mobility in the environment, which may promote water contamination. Fortunately, quinoline is readily degradable by certain microorganisms, such as *Rhodococcus* species Strain Q1, which was isolated from soil and paper mill sludge.

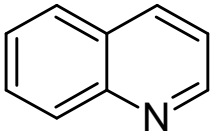
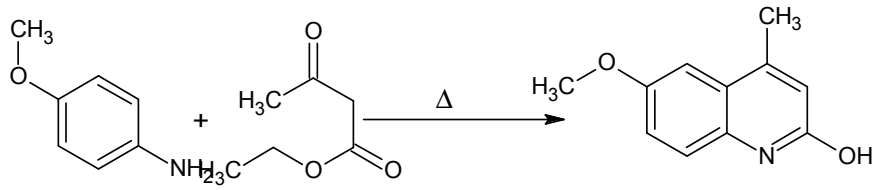


Fig. 1. Structure of quinoline.

Quinolines are known as benzopyridines because they have both, a benzene ring and a pyridine ring. Like benzene and pyridine, they are aromatic compounds. Quinoline undergoes electrophilic aromatic substitution on the benzene ring because benzene ring is more reactive than the pyridine ring toward electrophilic substitution. Substitution takes place primarily at C-5 and C-8. Nucleophilic substitution of quinoline undergoes at C-2 and C-4 because pyridine ring is more reactive than benzene.

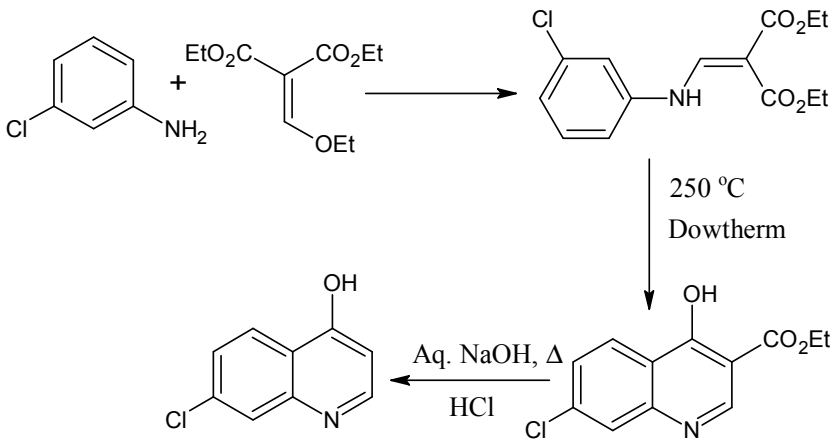
Synthesis of Quinoline Derivatives

In 1946 Campbell et al. [6] synthesized ethyl quininate from *p*-anisidine and acetoacetate by heating the reaction mixture. It is an easy method to synthesize 6-substituted quinoline derivative in mild condition as compared to other method.



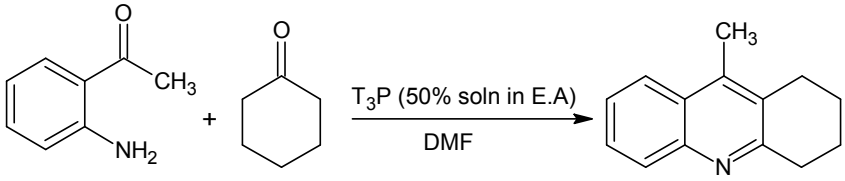
Scheme-1

Quinoline derivative with hydroxyl substitution at fourth position of the ring was synthesized by Charles et al, 1948 [7]. Here author used simple condensation followed by cyclisation reaction using dowtherm solvent at high temperature (Gould–Jacobs method).

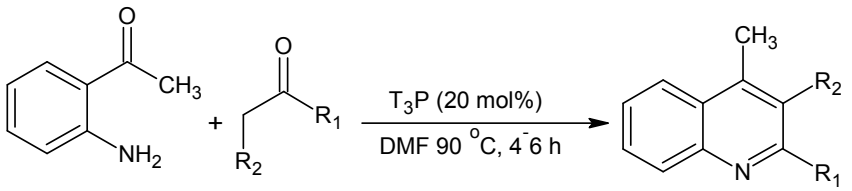


Scheme-2

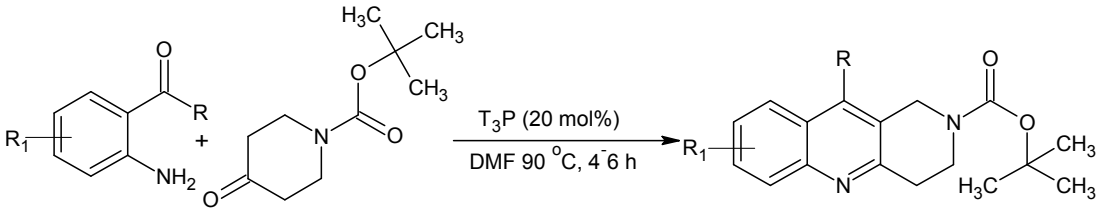
Quinoline can be easily synthesised by using different catalyst in mild conditions. Augustine et al. [8] synthesised quinoline derivative with mild reagent T3P (Propylphosphonic anhydride). This protocol is free from side reaction, such as self-condensation of ketones. It has tolerance for both acid sensitive and base sensitive functional groups with good yield.



Scheme-3

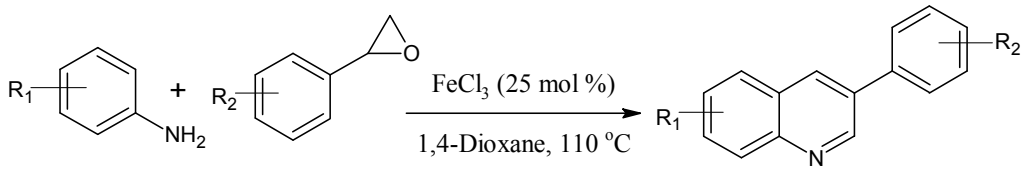


Scheme-4



Scheme-5

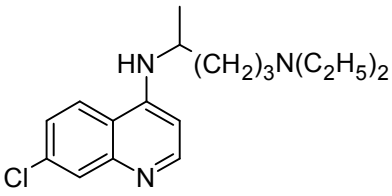
Recently, Zhang et al. [9] synthesized 3-arylquinolines from simple and readily available materials. The method used for synthesis is very simple, effective and using low cost catalyst ($FeCl_3$).



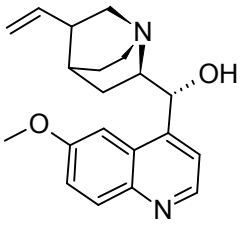
Scheme-6

Biological Importance of Quinoline

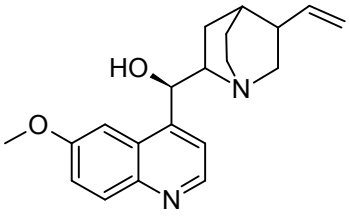
The quinoline scaffold is prevalent in a variety of pharmacologically active synthetic and natural compounds. The quinolines are historically most important antimalarial drugs ever used. Chloroquine, is the most famous drug of this group provided well-founded hopes for the eradication of malaria. The other known drugs from this family include Quinidine, Quinine, Ciprofloxacin, Grepafloxacin, Antrafenine, Saquinavir, Gemifloxacin, Topotecan, Balofloxacine, Levofloxacin. Drugs which are having quinoline as core molecule are given below.



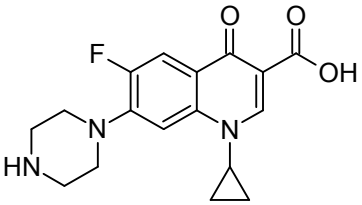
Chloroquine (antimalarial)



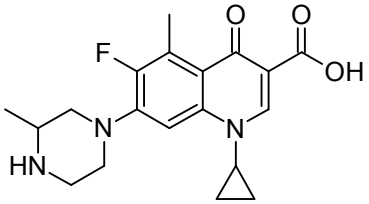
Quinidine (antiarrhythmic)



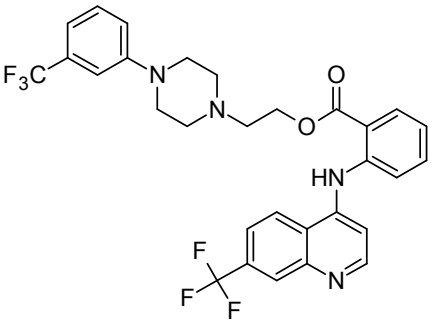
Quinine (antipyretic)



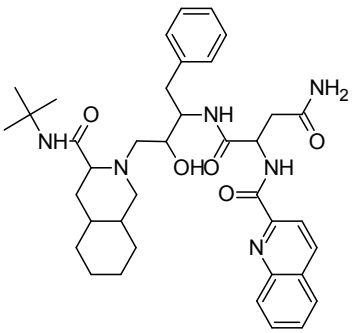
Ciprofloxacin (antibiotic)



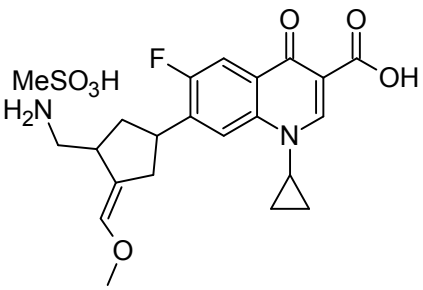
Grepafloxacin (antibacterial)



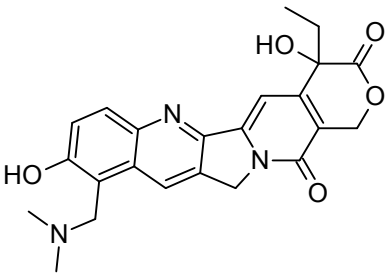
Antrafenine (analgesic)



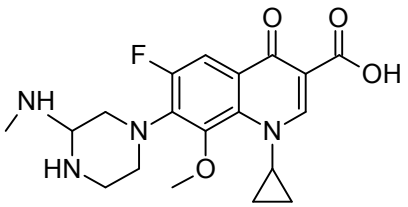
Saquinavir (antiretroviral)



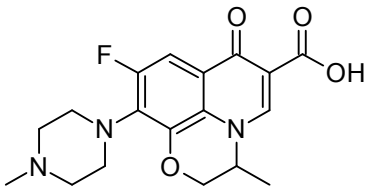
Gemifloxacin (antibacterial)



Topotecan (chemotherapeutic agent)



Balofloxacine



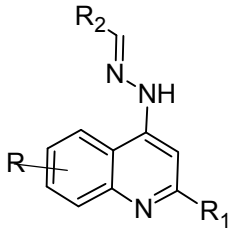
Levofloxacin (antibiotic)

Fig. 2. Structures of quinoline drugs.

In recent years, researchers are started derivatizing at different position of the quinoline ring with active pharmacophore to enhance the biological profile with respect to different applications. Some of the literature reviews are summarized bellow by focussed on position and core quinoline ring.

Biological Importance of 4-substitutedquinolines

Quinine is a natural white crystalline alkaloid having antipyretic, antimalarial, analgesic, and anti-inflammatory properties and having bitter taste. Chloroquine, a 4-substitutedquinoline, was first chemically synthesized in 1934, as a substitute for quinine. Chloroquine is selectively deposited in the food vacuole of the parasite, exerting its antimalarial effect by preventing the polymerization of the toxic heme. Chloroquine-resistant, antibiotic-resistant started appearing and now drug is virtually ineffective in most parts of the world. As Chloroquine resistance started, several efforts were initiated to develop new antimalarial, antibiotic drugs that target the resistant parasites. The focus of the research was in the direction of synthesis of side-chin modified of 4-substitutedquinolines and hybrid 4-aminoquinolines. Synthesis of hydrazones of quinoline moiety was reported by Savini et al, 2002 [10]. Newly synthesized compounds were screened for their antitubercular activity against *Mycobacterium tuberculosis* H37Rv. All the tested compounds in the series were shown to exhibit excellent inhibitory activity. It was noticed that introduction of 6-cyclohexyl, 7-methoxy, 7-ethoxy and 7-chloro substituents on the quinoline nucleus enhanced the antitubercular activity remarkably.

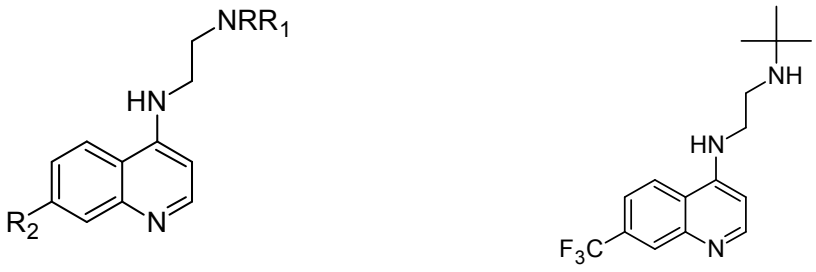


Where, R = H, CH_3, C_6H_5, R_1 = H, F, Cl, 5,7-Cl, OCH_3, OC_2H_5, C_4H_9, C_6H_{11}

R_2 = OCH_3,NO_2, furyl, pyrrolyl, 4-$N(C_2H_5)_2$-C_4H_4, naphthyl, 3,4-(OCH_2)-C_6H_3.

Fig. 3. Compound 1.

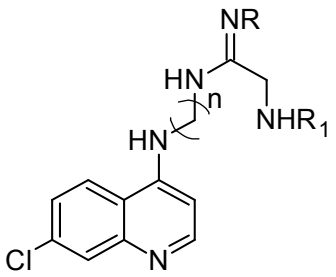
Stocks et al. [11] showed that the replacement of the diethylamino function with a metabolically stable t-Bu group or heterocyclic ring (piperidyl, pyrrolidinyl, and morpholinyl) in the short chain analogues led to a substantial increase in the antimalarial activity. The most promising analogue exhibited a 20-fold increase in potency against the chloroquine resistant strain with an IC_{50} value of 9.8 nM.



Where, R = H, Alkyl, R_1 = t-Bu, Alkyl, R_2 = Cl, CF_3.

Fig. 4. Compound 2.

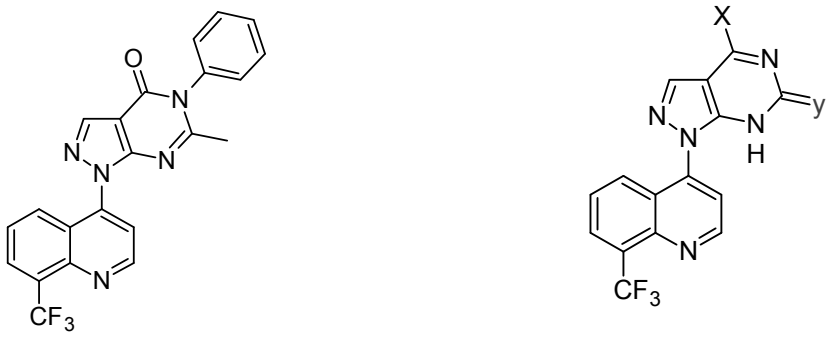
Solomon et al. [12] synthesized 4-aminoquinolines by selectively modifying the pendent amino group to facilitate the iraccumulation in the parasite food vacuole to achieve better interaction with the hematin leading to improved antimalarial activity. The compounds having the Boc group displayed MIC values ranging between 1.02 and 1.08 μM, were found to be more active than the corresponding amino compounds.



R = H, CH_3, CO_2t-Bu, R_1 = H, CO_2t-Bu, CH_3, n = 2, 3,4.

Fig. 5. Compound 3.

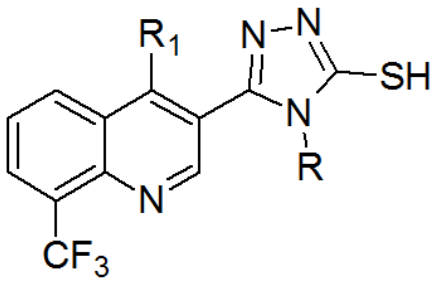
Holla et al. [13] reported the synthesis of some new pyrazolo[3,4]pyrimidine derivatives and its antimicrobial studies. Replacement of 1H of pyrazole of pyrazolo[3,4]pyrimidine ring system by some other bioactive moiety drastically alters its pharmacological properties. Introduction of a fluorine atom as the CF_3 group provides a more lipophilically and pharmacologically interesting compound compared to their non-fluorinated analogues.



X = NH_2, Y = O, S.

Fig. 6. Compound 4.

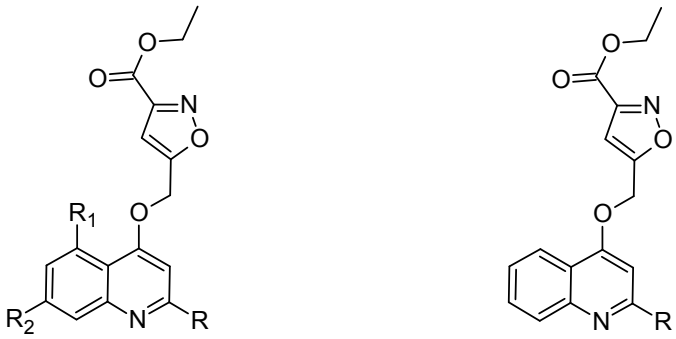
Eswaran et al. [4] reported synthesis and antimicrobial activity of new 1,2,4-triazole carrying quinoline derivatives using multistep reactions. The biological results revealed that cyclopropylamine, cychlohexylamine and morpholine at position 4 of quinoline enhanced antibacterial and antifungal activity.



R = Ph, -CH_2Ph, -CH_2CH_2OMe, R_1 = Substituted amines.

Fig. 7. Compound 5.

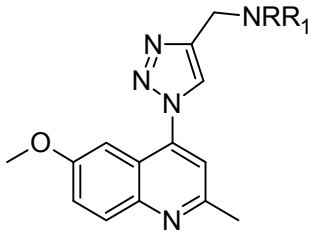
Lilienkampf et al. [14] identified a class of quinoline-isoxazole hybrid compounds with good anti-TB (Antitubeculosis) activity. Their investigation report revealed that most of the compound carrying C-2, C-7 trifluoromethyl quinolines and oxazoline at fouth position are better antituberculosis agents. According to authors, the isoxazole moiety played a significant role in the tuberculosis activity.



Where, $R/R_1/R_2$ = H, CF_3.

Fig. 8. Compound 6.

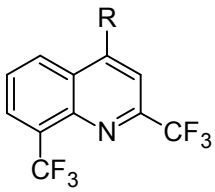
In the recent year Thomas et al. [15] designed, synthesized and evaluated antimicrobial activity of some new 1,2,3-triazole containing quinoline derivatives. The *in-vitro* preliminary antimicrobial screening revealed that, the presence of active groups like cyclopropyl, substituted piperazines, methoxy and fluoro has contributed significantly in enhancing the activity.



Where, R, R_1 = Alkyl amine.

Fig. 9. Compound 7.

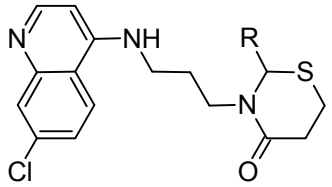
Meshram et al. [16] discribed synthesis of 2,8-bis(trifluoromethyl)-4-substituted quinoline derivatives and its anticancer activity. All the synthesized compounds were evaluated for their *in-vitro* cytotoxic activity. The results of their work suggest that hetirocylcic derivatives at fourth position are potent molecules.



Where, R = Substituted aryl, heteroaryl, 3-hydroxypiperidine, morpholine, benzimidazole.

Fig. 10. Compound 8.

Rudrapal et al. [17] synthesized and studied antimicrobial activity of some 3-(3-(7-chloroquinolin-4-ylamino)propyl)-1,3-thiazinan-4-one derivatives. They found that most of the tested compounds were useful microbial inhibitors. Results of antibacterial study indicate that aromatic bulky substituents have greater contributing effect than the aliphatic non-bulky group toward the antibacterial activity of the prepared 4-aminoquinoline derivatives.

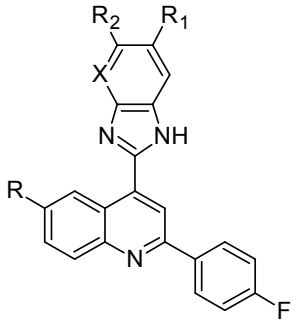


Where, R = 2-Fluorophenyl, 4-methoxyphenyl, 3-hydroxyphenyl, furan-2-yl-, ethyl, 4-

(dimethylamino)phenyl, 5-methyl-thiophen-2yl.

Fig. 11. Compound 9.

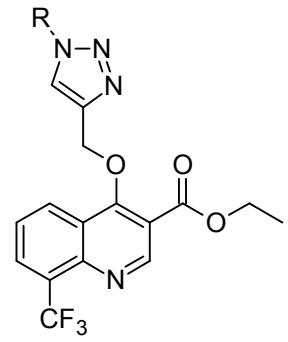
Quinoline incorporated benzimidazole derivatives were synthesized by Garudachari et al. [18] from substituted aniline and isatin through multi-step reaction. Final compounds were investigated for their *in-vitro* antimicrobial and antifungal activities by well plate method. Among the screened samples, dichlorosubstituted quinoline derivatives and nitrogene on the bezimidazole moiety showed excellent activity compared with other derivatives.



Where, R = H, F, Cl, R_1= H, Cl, OMe, R_2= CH, N.

Fig. 12. Compound 10.

Garudachari et al. [19] synthesized three series of 8-trifluoromethylquinoline based 1,2,3-triazoles derivatives by click chemistry approach. The synthesized compounds were characterized by spectral studies, single crystal X-ray analysis and screened for their antimicrobial activities. The *in-vitro* biological results showed that, increase of electron donating strength on the 1,2,3-triazole (alkyl chain and methoxy substitution) decreases antibacterial activity. On the other hand, introducing halogen or electron withdrawing phenyl ring on 1,2,3-triazole with trifluromethyl quinoline increases the antibacterial activity. The activity showed by the synthesized compounds were due to both 1,2,3-triazole and quinoline core rings.

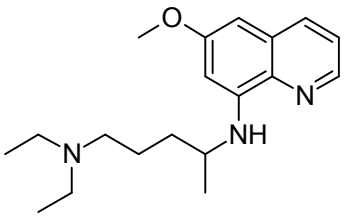


Where, R = Alkyl, benzyl, phenacyl.

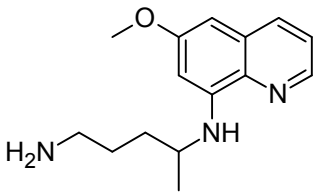
Fig. 13. Compound 11.

Biological Importance of 8-aminoquinolines

Pamaquine was synthesized in 1952, this 8-aminoquinoline was the first drug capable of preventing the relapses in *Plasmodium vivax* malaria. Toxicological concerns led to restrictions in the use of Pamaquine. Primaquine another 8-aminoquinoline derivative has been used since 1950's for the eradication of liver stages in course of *Plasmodium vivax* infections.



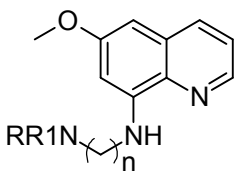
Pamaquine



Primaquine

Fig. 14. Compound 12.

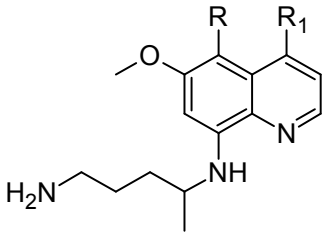
Syntheses of some 8-aminoquinoline derivatives were reported by Armer et al. [20]. Novel analogues were screened for both *in-vitro* and *in-vivo* anticoccidial activity. Pentyl group at 8th position of quinoline ring showed better anticoccidial activity. Other analogues were less potent *in-vitro* or *in-vivo* or both. Also, an exploration of the terminal nitrogen substitution has revealed the tetrahydropyran group to be optimal.



Where, R, R_1 = H, Alkyl, n = 1,2,3,4,5.

Fig. 15. Compound 13.

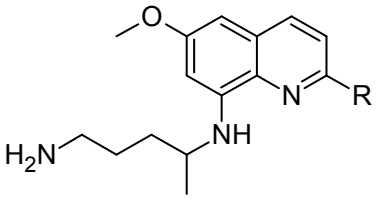
Several ring-substituted 8-aminoquinolines possessing remarkable antimalarial activities have been reported by Vangapandu et al. [21]. The substitution, R = C_5H_{11} and C_8H_{17} exhibited *in-vitro* and *in-vivo* biological efficacy higher than Chloroquine against both Chloroquine and Chloroquine resistant strains (IC_{50} of 9.4 and 9.7 ng/mL, respectively).



Where, R = C_2H_5, C_3H_7, C_4H_9, C_5H_{11}, C_6H_{13}, C_7H_{15}, C_8H_{17}, R_1 = C_2H_5.

Fig. 16. Compound 14.

Jain et al. [22] synthesized a series containing metabolically stable bulky alkyl groups at the C-2 position of the quinoline ring in Primaquine. The most promising analogue, *2-tert*-butyl primaquine displayed potent *in-vitro* antimalarial activity (IC_{50} = 39 ng/mL), superior to that of Chloroquine (IC_{50} = 113ng/ mL).



Where, R = H, $C(CH_3)_3$, 1-adamantyl.

Fig. 17. Compound 15.

Kaur et al. [23] reported synthesis and antimicrobial, antiprotozoal, antimalarial activity of bisquinoline analogues. The bisquinoline analogues exhibited promising *in-vitro* antimicrobial activities against a panel of pathogenic bacteria and fungi. The results of this study provide evidence that bis(8-aminoquinolines) are a promising class of antimalarial agents.

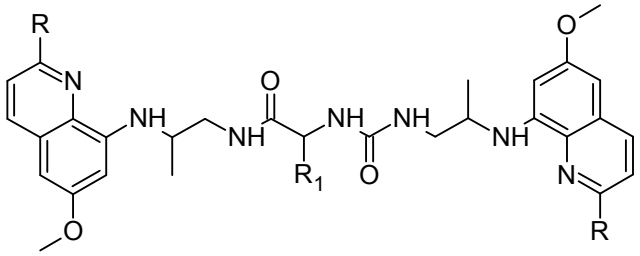
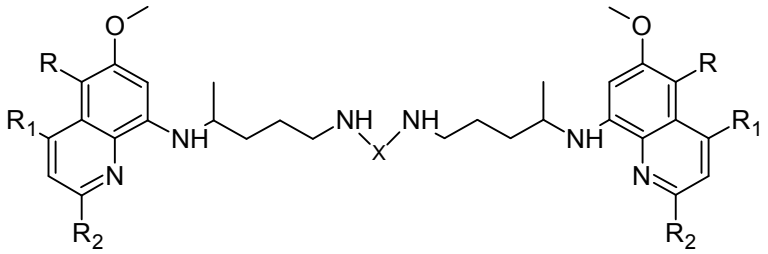


Fig. 18. Compound 16.

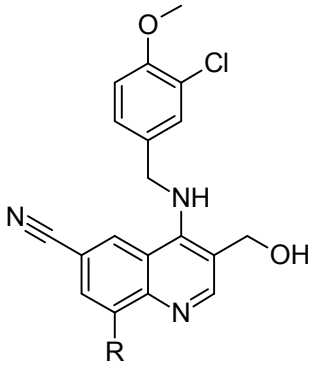


Where, R = H, OC_5H_{11}, R_1 = H, C_2H_5, R_2 = H, $C(CH_3)_3$

X = CO, CS, $COCH_2$, CONHCO, $COOCH_2$, COS, COCO, $CH_2CH_2NHCH_2CH_2$, Alkylaromatic.

Fig.19. Compound 17.

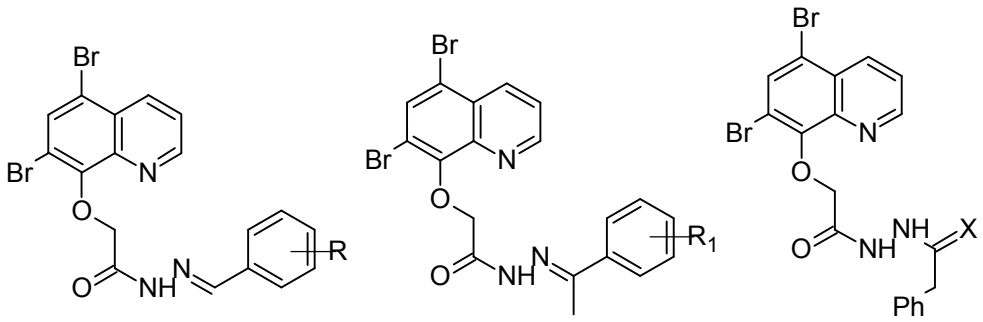
Fiorito et al. [24] synthesized potent quinoline derivatives for Alzheimer's disease. Among the synthesized compounds, cyclopropyl amine group at eighth position of the quinoline ring showed good pharmacokinetics profile.



Where, R = Cyclopropylamine, dimethylamine, ethylamine, morpholine, cyclopropyl.

Fig. 20. Compound 18.

Several derivatives of quinoline scaffold with a flexible, semi-flexible or rigid side chains at position 8 of the quinoline ring were synthesized by Arafa et al. [25]. Studied *in-vitro* activity versus the human colon cancer cell line HT29 and the human breast cancer cell line MDA-MB231. The derivatives with Schiff's base linkers showed excellent activity.

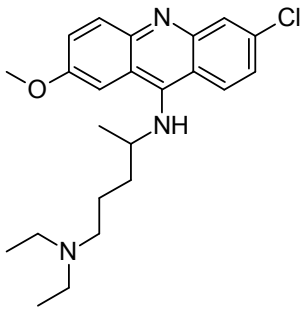


Where, R = H, 4-Cl, 4-Br, 4-NO_2, 3-OMe, 4-OH, 4-OMe, R_1 = H, 4-Br, 4-NO2, X = O, S.

Fig. 21. Compound 19.

Biological Importance of Ring Modified Quinolines

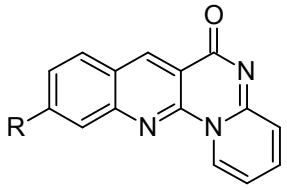
Quinacrine was initially approved in the 1930s as an antimalarial drug. In addition it has been used for treating tapeworm infections, giardiasis (an intestinal parasite) treatment.



Quinacrine

Fig. 22. Compound 20.

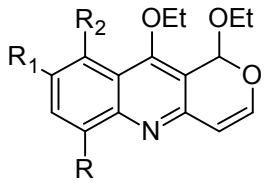
El-Sayed et al. [26] reported synthesis and antimicrobial evaluation of several quinoline and pyrimidoquinoline derivatives. The pyridine containing compounds were exerted strong antibacterial and antifungal activities, especially when a methoxyl group was located in the 7-position of quinoline nucleus.



Where, R = H, Me, -OCH_3.

Fig. 23. Compound 21.

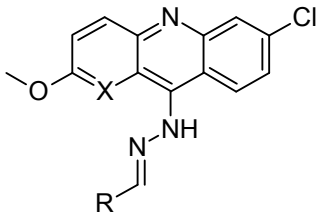
Synthesis of new 1,10-diethoxy-1*H*-pyrano[3, 3]quinolines and their antimicrobial studies were reported by Dhanabal et al. [27] . All the compounds exhibited moderate antibacterial activity. Interestingly the compound 6-methoxy substituted pyranoquinoline showed better activity than the standard Streptomycin in case of *Escherichia coli*.



Where, R = H, Me, R_1 = H, -OMe, Cl, R_2 = H, Me.

Fig. 24. Compound 22.

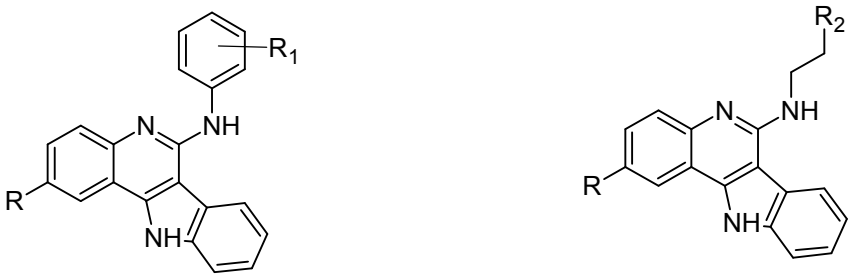
Fattorusso et al. [28] evaluated N_2-acrydinylhydrazones for antiplasmodial activity. The imidazoles containing hydrazone derivatives showed better activity against the W2 strain and D10 strain (IC_{50} = 30.8 and 26.9 nM respectively).



Where, R = Imidazoles and Substituted phenyl, X = CH, N.

Fig. 25. Compound 23.

Antiproliferative evaluation of certain indolo[3,2]quinoline derivatives were reported by Lu et al. [29]. The introduction of a hydroxyl group at the anilino-moiety resulted in the enhancement of antiproliferative activity in which the activity decreased in an order of *para*-OH > *meta*-OH > *ortho*-OH. The C_6 alkylamino-substituted indolo[3,2]quinoline derivatives, exhibited comparable antiproliferative activities against all tested cancer cells.



Where, R = H, F, R_1 = H, 4-OH, 3-OH, 2-OH, 4-Me, R_2 = alkyl amine, aromatic alkyl amine.

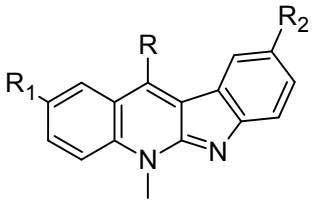
Fig. 26. Compound 24.

Series of 4-alkoxylated and 4-aminated benzofuro[2,3]quinoline derivatives was synthesized, evaluated for their anti-TB and cytotoxic activities [30]. Among the tested compounds, methoxybenzofuro[2,3]quinoline, methylamino-benzofuro[2,3]quinoline, dimethylamino benzofuro [2,3] quinoline, exhibited significant activities against the growth of *Mycobacterium tuberculosis* (MIC values of <0.20 mg/mL).

Where, R = Me, Et, Ph, Ph-4-COMe, R_1 = H, Me, R_2 = H, Me, Ph, Ph-4-COMe, Ph-4-OMe, Ph-3-OMe.

Fig. 27. Compound 25.

Lu et al. [31] described the synthesis, and *in-vitro* and *in-vivo* antimalarial evaluations of certain ester modified Neocryptolepine (5-methyl-5H-indolo[2,3-b]quinoline) derivatives. Modification was carried out by introducing ester group at C-2 and C-9 position of Neocryptolepine core. All the tested compounds showed higher activity than the well-known antimalarial drug Chloroquine.

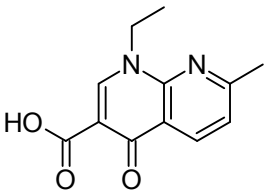


Where, R = Alkylamine, N-substituted alkylamine, alcohol, morpholine, R_1 = H,CO_2Me, Cl, Br, R_2 = H, CO_2Me, Br.

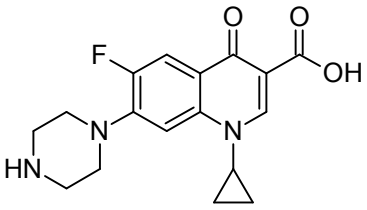
Fig. 28. Compound 26.

Biological Importance of Quinolones

The quinolones are a family of synthetic broad-spectrum antibiotics. The first generation of the quinolones begins with the introduction of Nalidixic acid in 1962 for treatment of urinary tract infections in humans. Nalidixic acid was discovered by George Lesher and co-workers.



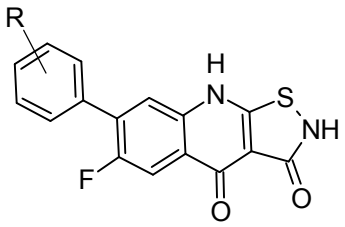
Nalidixic acid



Ciprofloxacin

Fig. 29. Compound 27.

Wiles et al. [32] reported synthesis of isothiazoloquinolones containing functionalized aromatic hydrocarbons at the 7-position and its antibacterial activity. The activity of 3-substituted analogues against MRSA was greater than that of the corresponding 4-substituted analogues.

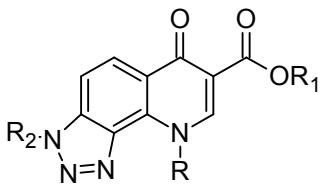


Where, R = H, $NH_2(CO)$, AcNH, Ac, NC, $NCCH_2$, F, OH, OMe, NH_2, $OHCH_2$, 4-OH-2,5-Me,

NH_2CH_2, $NH_2(CH_2)_2$, NMe, 3-NH_2-4-Me, 3-NH_2-4-F, Piperidinyl.

Fig. 30. Compound 28.

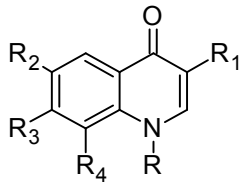
Synthesis of triazole containing quinolones was reported by Carta et al. [33]. The newly synthesized compounds were screened for their antitubercular activity. N-methyl quinoline ethylester are showed MIC_{90} values in the range 0.5-3.2 μg/mL, while other compounds were inactive at MIC_{90} = 32 μg/mL.



Where, R = H, N-CH_3, R_1 = H, C_2H_5, R_2 = H, C_2H_5.

Fig. 31. Compound 29.

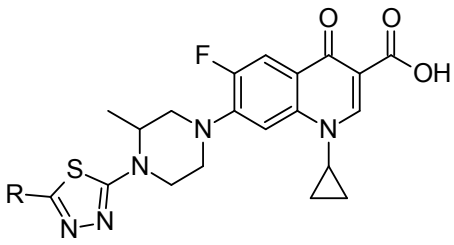
Ma et al. [34] reported synthesis, *in-vitro* antitrypanosomal and antibacterial activity of phenoxy, phenylthio or benzyloxy substituted quinolones. The compound having 7-monosustitution showed significant antibacterial activities (MIC < 25 μg/mL). Most 7,8-disubstituted quinolones exhibited significant inhibitory activity against antitrypanosomal agents.



Where, R = C_2H_5, Pr, $CH{=}CH_2$, R_1 = COOH, COOMe, COOEt, $CONH_2$, CN, R_2 = F, 4-CH_3-PhO, PhS, $PhCH_2O$, R_3 = 4-CH_3-PhO, 2,4-Dichloro-PhO, PhO, 4-F-PhS, 4-Cl-PhS, 4-CH_3-PhS, $PhCH_2O$, R_4 = 4-CH_3-PhO, 2,4-Dichloro-PhO, 4-OH-PhO, PhO, PhS, 4-F-PhS, 4-Cl-PhS, $PhCH_2O$, F.

Fig. 32. Compound 30.

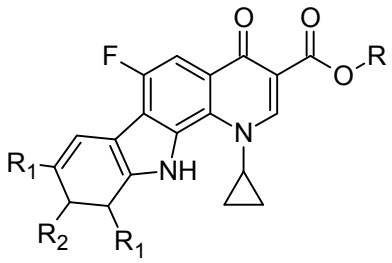
Antibacterial evaluation of certain nitroaryl thiadiazole-gatifloxacin hybrids were reported by Jazayeri et al. [35]. Among synthesized compounds, nitrofuran-1,3,4-thiadiazole moiety attached to the piperazine ring at C-7 position exhibited more potent inhibitory activity against Gram-positive bacteria including *Staphylococcus epidermidis* (MIC = 0.0078 mg/mL), *Bacillus subtilis* (MIC = 0.0039 mg/mL), *Enterococcus faecalis* (MIC = 0.125 mg/mL) and *Micrococcus luteus* (MIC = 0.125 mg/mL).



Where, R = Nitrofuran, nitrothiophene, nitrophenyl, N-methyl-nitroimidazole.

Fig. 33. Compound 31.

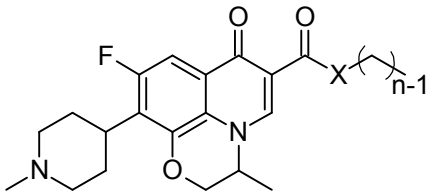
A series of tetracyclic fluoroquinolones were synthesized by Al-Trawneh et al. [36]. All synthesized derivatives were tested for their *in-vitro* antimicrobial and antiproliferative activity. The fluoroindole fused quinolone compound emerged as the most active antibacterial compound against multidrug-resistant *staphylococci* and the most potent antiproliferative compound against MCF-7 cells.



Where, R = H, Et, R_1 = H, Me, OMe, F, R_2 = H, Me.

Fig. 34. Compound 32.

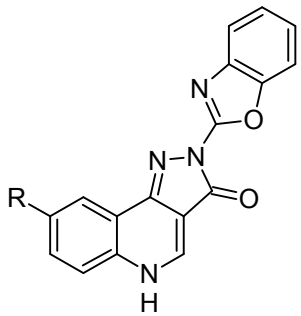
A convenient route for the synthesis of some acyloxymethyl esters and carboxamides of Levofloxacin (LV) with modulated lipophilicity was described by Korolyov et al. [37]. All newly synthesized compounds were evaluated *in-vitro* antitumor activity against five human cancer cell lines. The most efficient LV derivatives (ester and amide) displayed IC_{50} values in 0.2-2.2 μM range.



Where, X = O, NH, n = 4-15.

Fig. 35. Compound 33.

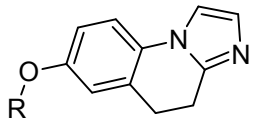
In the recent years, Reis et al. [38] reported synthesis and anticancer activity of 2-(benzothiazol-2-yl)-8-substituted-2H-pyrazolo[4,3]quinolin-3(5H)-ones. The *in-vitro* anticancer activity of the synthesized compounds revealed, methyl and bromo substitution at C-6 position of quinoline showed considerable effect against three cancer cell lines. IC_{50} value of 2.3 mg/mL against breast cancer (MDA-MB-435), colon (HCT-8) and central nervous system (SF-295) cell lines (IC_{50} values of 4.1 and 4.5 mg/mL, respectively).



Where, R = H, CH_3, Br, OCH_3, Cl, NO_2.

Fig. 36. Compound 34.

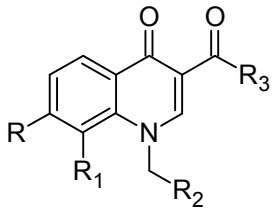
Sun et al. [39] synthesized 7-alkyloxy-4, 5-dihydro-imidazo[1,2-a]quinoline derivatives and studied their antimicrobial activity. Most of the compounds exhibited potential antibacterial activity against gram-negative and gram-positive bacteria. The compound (7-heptyloxy-4,5-dihydro-imidazo[1,2-a]quinoline) showed excellent activity than that of reference agent Ciprofloxacin.



Where, R = Aryl, alkyl group.

Fig. 37. Compound 35.

Three series of some new trifluoromethyl substituted quinolone derivatives were synthesized by Garudachari et al. [40]. The synthetic routes and purification methods were stabilized for regioselective synthesis by approaching two different routes. The compounds containing trifluoromethyl group at seventh and eighth position of quinolone, ethylester, hydrazide pharmacophore and at the third position of N-alkylatedquinolone core moiety showed enhanced antibacterial, antifungal activity.



Where, R/R_1= H, CF3, R_2= proparzyl, 2,4-dichlorophenyl, 4-cyanophenyl , R_3= OH, NH_2NH_2,

ethyl, proparzyl, 2,4-dichlorophenyl, 4-cyanophenyl.

Fig. 38. Compound 36.

Conclusion

Quinoline drugs have caused a dramatic change not only of the treatment of infectious diseases but of a fate of mankind. Because of its wide range of application in the medicinal chemistry, quinoline derivatives got primary position in the heterocycles. Quinoline core ring can be easily synthesized by using simple aniline derivatives from various well-known reactions. Depend on its various pharmacological application, researchers are concentrated towards the modification of quinoline core ring with active pharmacophores at different position. The development of potent and effective pharmaceutical agent is most important to overcome the emerging multi-drug resistance strains of pathogens. In this way quinoline is an important heterocyclic compound, which played an important role in developing new antimicrobial, anticancer, antimalarial, anticonvulsant and antituberculosis drugs. As regards the relationships between the structure of the quinoline scaffold and detected biological properties, it showed that heterocyclic substitution at different position of the quinoline

are ideally suited for obtaining more efficient pharmaceutical compounds. Still, there is a huge scope to implement quinoline derivatives by modifying their active pharmacophores, in order to develop further applications in medicinal chemistry.

Acknowledgement

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Congenital Heart Diseases and Biotechnology: Connecting by Connexin

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Keywords: connexin, heart development, cardiomyocyte, congenital heart disease, gap junction channel, gene delivery, peptidomimetics

Abstract. Heart development is a precisely harmonized process of cellular proliferation, migration, differentiation, and integrated morphogenetic interactions, and therefore it is extremely vulnerable to developmental defects that cause congenital heart diseases (CHD). One of the major causes of CHD has been shown to be the mutations in key cardiac channel-forming proteins namely, connexins (Cxs). Cxs are tetra-spanning transmembrane proteins that form gap junction channels and hemichannels on cellular membrane. They allow passage of small molecules or ions between adjacent cells or between cells and the extracellular environment. Studies have revealed that the spatiotemporal expression of Cxs mainly, Cx31.9, Cx40, Cx43, and Cx45 is essentially involved in early developmental events, morphogenetic transformations, maturation, and functional significance of heart. Our lab and others have shown that mutations in gap junction proteins could result in impaired trafficking, misfolding, and improper channel function of these proteins. It has also been shown that differential expressions of cardiac Cxs are associated with pathophysiological conditions of heart. Collectively, these conditions are coupled with abrogated or modified functionality of relevant channels in cardiac tissue, which are associated with many pathological situations, including CHD. Since CHD are a major cause of morbidity, therefore recovery of such kind of heart defects associated with Cxs is extremely important but remains highly challenging. In this review, we will summarize the role of Cxs in development, morphogenesis, maturation, normal function, and pathology of heart, and propose possible bioengineering techniques to recover defects in cardiac tissues related to the modified functions of Cxs.

Introduction

Congenital heart diseases (CHD) are collectively one of the major causes of morbidity in newborn with a prevalence of 1% among the total live births [1]. It is highly likely that the number of occurrence could be even significantly higher should there be perfect monitoring and detection technology and system effectively applied [2]. Approximately 90% of children with life-threatening CHD who have access to surgery survive to adulthood [3]. The pathology of CHD spans diverse symptoms ranging from moderate defects like atrial septal defects (ASDs) or ventricular septal defects (VSDs) to severe heart diseases like hypoplastic right or left heart syndrome, Morbus Fallot, Ebstein's malformation, and transposition of the great arteries (TGA) [4,5]. CHD tend to develop at increased frequency within the familial groups, and with a considerably higher risk for the first

degree relatives including descendants and siblings [6]. For twins, if either of the twins is affected by CHD then the prevalence for the CHD is very high for the other twin, and the probability is significantly higher for monozygotic twins over a dizygotic twin siblings. It was further observed that if both twins of a monozygotic sibling exhibit any CHD then the possibility of developing the same cardiac malformation or malfunction in both of them is more than 90% [7]. Although unknown numbers of premature failing fetuses are also linked with CHD-related heart failure, there is no clear statistical information regarding the incidence of such type of heart failure due to technical and ethical concerns [8]. Therefore, early detection of prevalence of CHD, and recovery of such defects in fetus are very pragmatic challenge to overcome.

Different animal models including mouse, chicken, and fish have been exploited to reveal the secret genetic factors associated with CHD. Studies on these animal models have produced significant amount of information for the causes and management of CHD, and many of these information are believed similarly, at least partially if not completely, responsible for CHD in human [4,9-11]. Despite much information on single nucleotide polymorphism (SNP), unusual gene expression, chromosomal anomalies, and abrogated or modified gene function related to CHD in model animals, precise genetic factors related to the development of CHD in human still remains elusive.

Over the recent decades, researches have attempted to understand these dissimilarities through typical genome-wide analyses of uncommon Mendelian CHD families and by sequencing contender genes in CHD cohorts. Studies revealed that human CHD-related mutations affect multiple factors that control cardiac development and such mutations often alter expression of multiple genes or gene products [4,9-11]. It was also understood that identical pathogenic mutations related to CHD, for example the Holt-Oram syndrome or Noonan syndrome, develop diverse type of distinct cardiac malformations indicated that CHD is multifactorial event [10]. Recent advances in genomic and proteomic technologies including single nucleotide polymorphism (SNP) arrays, next-generation sequencing, high throughput screening, MALDI-TOF, and copy number variant platforms are speeding-up the discovery of genetic factors responsible for CHD. Reportable that such kind of new technologies and approaches are suitable for studying sporadic cases of CHD, which is the most frequent appearance of CHD [10]. These studies have been revealing new information on CHD in human, and providing scientific evidences for the previously observed phenomenon in model animals.

Heart development process has been elucidated using model animals, and has been found to be a highly synchronized multistep process that starts at the early stage of embryogenesis.This is orchestrated by precise spatiotemporal expression and function of many gene products. Commitment of cardiogenic cells from mesodermal cells is believed to be initiated by endodermal signalling factors such as bone morphogenetic protein (BMP), basic fibroblast growth factors (bFGF), and int/wingless (Wnt) proteins, which signals through cell-surface receptors and triggers the relevant signaling cascades to execute the transformation process [12-14]. The cardiogenic cells first appear at the both side of the mid-line, and then migrate to form cardiac crescent. During the second stage of heart development process these cells further migrate towards the ventral midline and fuse to form primitive heart tube. Heart starts beating at this stage and the heart tube undergoes further morphogenetic maturation to form functionally matured heart [15]. The whole process of embryonic heart development and maturation are controlled by an array of genes, including (1) transcription factors like GATA4, Nkx2.5, TBX5 etc., (2) structural genes like cardiac troponin T, cardiac myosin etc., (3) autocrine and paracrine factors like natriuretic peptide (NP) A and NPB etc., (4) cell-connecting genes like cadherins, and (5) cellular channel-forming genes like connexin (Cx) proteins [4,10,12,14]. In this article we will focus on the roles and regulation of Cx and their significance on cardiac development and physiology. In addition, we will discuss as to how biotechnology can be applied to recover CHD by exploiting Cx-gene products.

Forms and Functions of Connexin

Cxs are integral membrane proteins having four transmembrane domains (M1 – M4), one intracellular loop, two extracellular loops (E1 and E2), and short cytoplasmic N-terminal and long C-terminal regions (Fig.1, A). Generally, Cx family members form homo-hexamers called connexons or hemichannels in the Golgi apparatus. Connexons are then sorted and transported to the plasma membrane as connexon hemichannel. Cell-surface connexons have a possible role in establishing communication conduits between the cytosol and the extracellular environment.

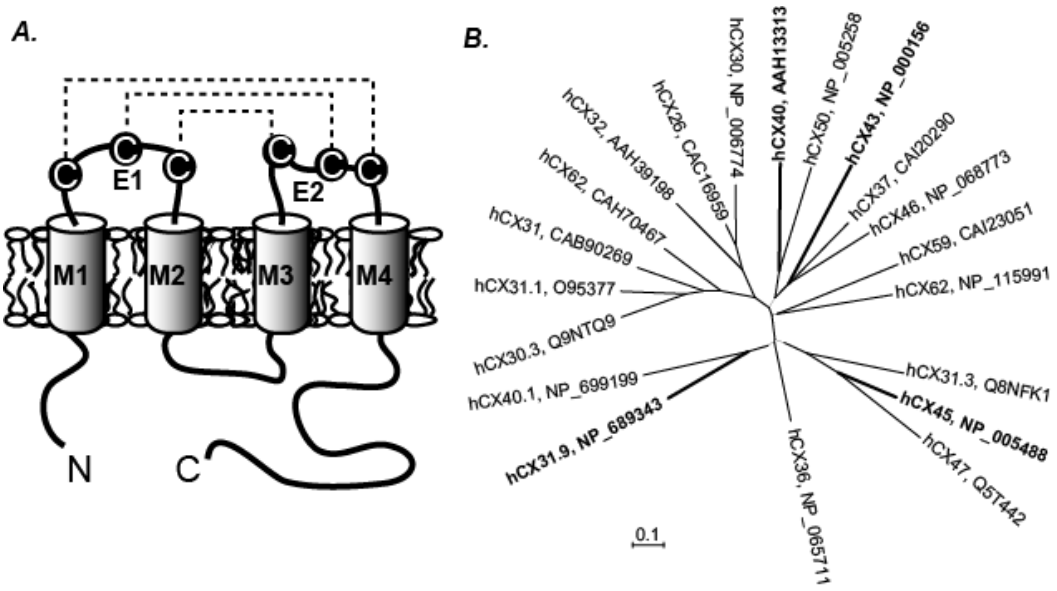

Fig. 1. Schematics and phylogenetic relationship of human Cx. (*A*), The schematic of a single Cx protein is shown. Conserved extracellular Cys rsidues are indicated with C. Cys-Cys di-dulfide linkages are shown with dotted lines. M1-M4, transmembrane domains, E1 and E2, extracellular loops, N and C denote respective terminal of the protein. (*B*), the phylogenetic locations of 20 human Cx proteins are shown. Respective accession numbers are shown adjacent to the relevant Cx. Cardiac connexins are shown in bold face; scale in centi morgan.

Two homomeric connexon hemichannels from apposing cells further dock on each other to form a gap junction channel that holds adjacent cells together at a distance of 2-4 nm apart [16- 20]. A schematic of constitutive pathway for Cxs is shown in Fig. 2. A heterotypic gap junction channel may form when two homomeric connexons (each consisting of the same connexins) pair up. A heteromeric gap junction channel may also form when two heteromeric connexons (each consisting of different Cxs)join. In some cases two or more connexins can selectively intermix to form heteromeric connexons [21]. Cell surface connexons or hemichannels may have possible role in establishing communication conduit between the cytosol and extracellular milieu. However, a connexin channel allows export or import of small molecules lower than 1kD between two adjacent cells; ATP, Ca^{2+}, glutamate, NAD^{+} etc. are some of the experimentally established natural substrates of Cx hemichannels/channels [22].

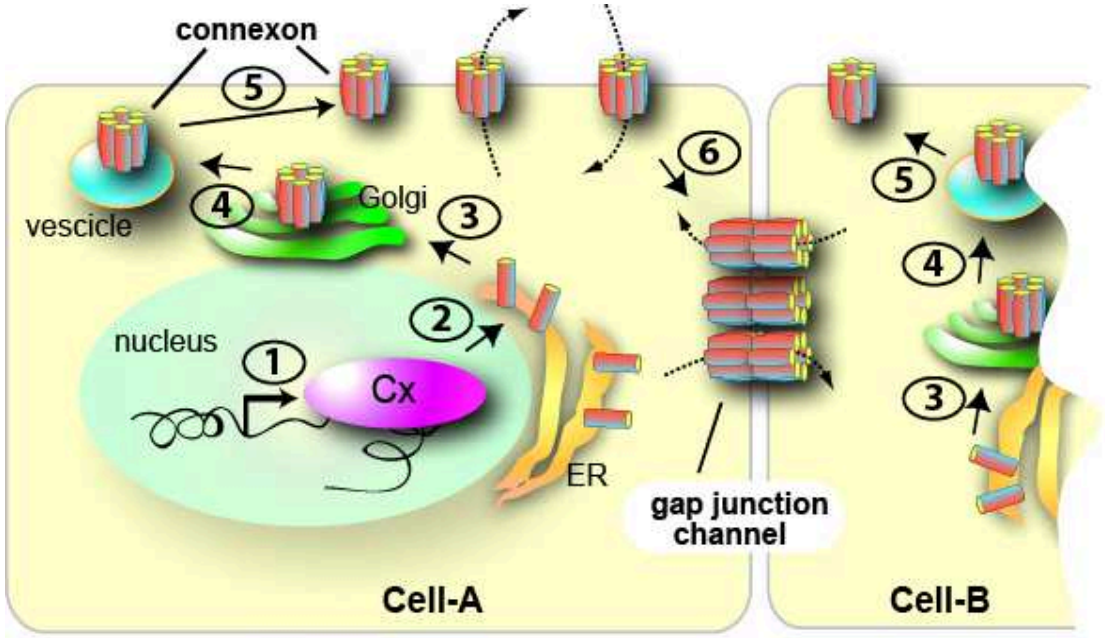

Fig. 2. Biogenesis of Cx.(1) The Cx gene is transcribed inthe nucleus, and (2) Cx proteins exist as monomer in the ER. (3) During the transition through the Golgi apparatus, they form hexameric connexons. (4 and 5) Connexons are then targeted to the plasma membrane *via* vescicular trafficking, and become functionally active as hemichannels. (6) Two apposing connexons from neighbouring cells join to form gap junction plaques.

Complete Cx gene families have been identified in many vertebrates including human, mouse, and zebrafish with 21, 20, and 37 genes, respectively, indicating significant diversity in form and function of gap junction channels [23,24]. Cxs are named after species and by the theoretical molecular weight in kD of the relevant monomeric protein. For example, mCx43 indicates that it is a mouse connexin with molecular weight of 43 kD. All Cxs have three conserved cysteine(Cys) residuesin each extracellular loop, which form intramolecular disulphide linkage (i.e., the first loop: $C\text{-}X_6\text{-}C\text{-}X_3\text{-}C$ and the second loop: $C\text{-}X_5\text{-}C\text{-}X_5\text{-}C$).These Cys residues form intramolecular disulphide linkages to connect the extracellular loops of apposingconnexons of neighboring cells during the docking process of connexons, whichis required for functional gap junction channel formation. These proteins are generally expressed as non-glycosylated forms, which facilitatesCx molecules to come closer to form hexameric connexons as well as dodecameric connexin channels [18,25].

Cxs shares a high amino acid sequence similarity in the transmembrane domains and extracellular loops, and have a greater degree of diversityin the cytoplasmic loop and C-terminal tail. The C-terminal tail contains various consensus sequences for phosphorylation by a number of protein kinases, such as protein kinase A (PKA), protein kinase B (PKB/Akt), protein kinase C (PKC), protein kinase G (PKG), mitogen-activated protein kinase (MAPK), cyclin kinase 1, cyclin-dependent kinase 2 (Cdc2), casein kinase 1 (CK1), sarcoma Rous kinase (src), and p34 (cdc2)/cyclin B kinase [18,26,27]. These kinases have been suggested to be intensely involved in the regulation and biochemical efficacy of Cx. Recent crystal structure information and mutagenesis studies revealed that the conducting channel region is formed by multiple domains of Cx protein:the N-terminal M1 and E1 domains contribute in the construction of a channelpore; the transmembrane

domains help in oligomerization; the E1 and E2 domains directly take part in docking process; and the intracellular regions regulate the pore-gating function as well as interact with partner proteins [28-30]. By default, Cx hemichannels open to facilitate intercellular communication and are regulated to close in response to the specific cellular stimuli, for example ion-challenged physiological conditions [31]. However, several Cx hemichannels remain close under normal physiological conditions and open in response to stress factors, such as sudden mechanical shock and specific physiological signals [32, 33]. In fact, hemichannel properties are being studied in laboratory setting by exploiting the fact that sudden mechanical shock, like dropping of liquid from certain height, can open Cx hemichannels located on the cell surface. Reportable that Cx hemichannels do not only express on the plasma membrane of cell but they can also express in intracellular membrane, as exemplified by the mitochondrial membrane localization of Cx43 [34].

Connexins in Heart Development and Function

The conserved pattern of heart development creates the scope to exploit the model animals (mouse, zebrafish, chicken etc.) to discover the novel genetic factors linked with vertebrate cardiogenesis [4, 14, 15, 35]. In recent years, increasing evidences in model animals suggested that Cxs, have important roles in cardiogenesis and heart function. Freeze-fracture electron microscopy revealed that as many as 200,000 gap junction channels can be formed at the cardiac intercalated disc suggesting significant role of these channels in heart function [36]. The spaciotemporal expression pattern of different Cx genes in cardiac tissues indicates their independent functional significances. In mouse heart, at least seven Cx gene products are expressed at different time and space:Cx30, Cx30.2, Cx37, Cx40, Cx43, Cx45, and Cx46 [5,37]. A numbers of knock-outs (KO), heart-specific KO, conditional KO, double KO, knock-ins (KI), and overexpression studies have been accomplished on mouse and zebrafish models to understand the roles of the cardiac-Cx in relevant development, morphogenesis, and function [5,37]. Different areas of heart show specific expression profile of Cx gene products with significant similarities, but not completely the same, between model animals and human, whichprovides substantial information to be used for human [11,15,16,38-42]. In human, cardiac gap junctions are comprised of generally five Cx isotypes namely, Cx31.9, Cx37, Cx40, Cx43, and Cx45 [5,25,38,40,43,44]. Each cardiac Cx has a different spatiotemporal expression pattern but some heart cells coexpress multiple Cxs during cardiac development, morphogenesis, and maturation and function suggesting cooperative activities of relevant Cxs in these cells [37]. In this article we will emphasize on the major cardiac connexins in relation with proper development and functional significance of vertebrate heart.

Connexin 43. Cx43 is the most widely studied connexin gene. In mouse adult heart, Cx43 is the dominant gap junction protein in ventricles, as well as highly expressed in atrial myocytes along with Cx40 [37]. Cx43 protein has been found to be localized in the interventricular septum but not observed in the cardiac conduction system [37]. It has been reported that the expression of Cx43 is suppressed by the transcription factor Tbx18 in sinoatrial (SA) node, whereas Tbx2 and Tbx3 suppress it in atrioventricular (AV) node [37] suggesting localized regulation of Cx43 in cardiac tissues. During the mouse embryonic development, the first cardiac expression of Cx43 has been detected in both ventricles at 9.5 day post-coitum (dpc); specifically, higher level of expression was detected in the trabeculated area of the ventricle at 12.5 dpc [40,45]. At a later time point, the expression of Cx43 spreads in the whole atrium and to the septum and is thereafter steadily expressed in adult heart [40,45]. Although the expression pattern of Cx43 in human developing heart has not been thoroughly examined, Cx43 is not highly expressed in human atrium, which is in contrast to mouse Cx43 [5,37,40,45].

It has been reported that Cx43 has a very short half-life of about 2 – 3 h [46]. This information indicates that long term adaptation of gap junction channels constituted of Cx43 to environmental or specific physiological conditions might be extremely critical for specific activities of relevant cardiac cells in respect of development and function. It has been shown that cyclic mechanical stretch redistributed Cx43 gap junction channels from a circumferential localization to polar localization in

cells within 24 h; such kind of polar distribution was lost after withdrawal of stretch [47]. This finding revealed that cells can execute redistribution of Cx proteins in response to extracellular stimulants, and suggested their multifunctional involvement in development and maturation of relevant cells or tissues.

Cx43 homozygous knock out ($Cx43^{-/-}$) mice die shortly after birth due to respiratory failure caused by a right ventricular outflow-tract obstruction [48]. Abnormal development of heart was observed in these mice during the looping phase at 10 dpc [49]. It has been proposed that improper and inefficient migration of cardiac neural crest cells are responsible for developing truncus arteriosus communis (TAC), one of the severe CHDs [50,51]. Atypical p53 activation in $Cx43^{-/-}$ mice causes apoptosis of primordial germ cells, which in turn produces outflow tract defect in these animals due to inefficient migration of specific cells to the neural crest crescent [50,51]. $Cx43^{-/-}$, as well as $Cx43^{+/-}$ mice developed with abnormal patterning of principal coronary arteries. Later in has been shown in mouse that alteration in Cx43 gene products resulted conotruncal heart defects with patho-morphology of the right ventricle, thinning of myocardium and narrowing of right ventricular outflow tract [5,37]. Interestingly, overexpression of Cx43 also developed cardiovascular abnormalities in mice indicating that a tight control of regulated expression of Cx43 is critical for the proper development of vertebrate heart [52]. Unlike $Cx43^{-/-}$ mice, $Cx43^{+/-}$ mice can survive and grow to their adulthood despite approximately 50% lower expression of Cx43 protein [53].

In 1995, by analyzing Cx43 mutations in patients with heart malformations and functional defects, Britz-Cunningham et al. found that missense mutations in the C-terminal of Cx43 protein affected several consensus phosphorylation sites [54]. They further demonstrated that the mutations altered phosphorylation of Cx43 protein by PKA and PKC, which are critical for intracellular interaction of Cx43 [54]. This study opened-up a new possibility whether mutations in Cx is responsible for cardiac malformation; soon after many studies provided important information regarding this issue. It has been reported that a glycine to serine at amino acid position 60 (G60S) of Cx43 acts in a dominant negative manner that causes oculodentodigital dysplasia and other cardiac defects including reduced cardiac function and patent foramen ovale [37,55-57]. The reason behind the modified function of Cx43 was shown to be associated with impaired trafficking of mutant Cx43 to the intercalated disc region. Two other mutants namely, G138R and I130T, have been shown to be associated with altered cardiac structure and function, which lead to oculodentodigital dysplasia phenotype [55,58]. G138R-Cx43 expression level was not altered but it developed a loss of phosphorylation to the relevant location of the protein. Yu et al. has shown that N-ethyl-N-nitrosourea- (ENU) mediated mutagenesis in mice that specifically substituted a G to A in the Cx43 gene, which generated a prematured truncated protein that was responsible to produce conotruncal malformation and coronary aneurysms [59]. A study on human fetal hearts (collected from aborted fetus where heart was affected by double outlet right ventricle defect; DORV) focusing on the role of possible mutations in Cx43 in human heart development, reported eight point mutations in the intercellular C-terminal region closer to the 4th transmembrane domain (M4) of Cx43 protein [60]. Particularly, one missense mutation that produced P238L was thought to limit Cx43 degradation. Since the correct amount of Cx43 protein is critical for proper heart development therefore it has been proposed that this mutation is responsible for developing DORV defect [60]. Recent study in 400 Chinese children confirmed the initial observation that mutations in C-termius of Cx43 might be associated with developmental defects of heart [61]. Most of the subjects included in the study had been suffering from DSDs or ASDs or Morbus Fallot. Interestingly, three missense mutations in the C-terminal tail of Cx43, which are consensus site for phosphorylation, were detected in all patients suffering from mentioned CHDs suggesting such mutations might have significant roles on the normal structure and function of Cx43 protein that ultimately developed CHDs in the experimental patients. However, there is a contrasting report as well indicating such kind of conclusion is difficult to make [62]. Another study in Morbus Fallot affected patients revealed that Cx43 protein is distributed in a circumferential pattern in cardiac cells compare to the polar distribution for normal condition [47]. Moreover, it was found that the expression level of Cx43 protein was significantly

lower in the above mentioned patient group compared to the control group, suggesting a critical role of Cx43 protein in proper heart development and CHDs.

Cx43 forms channels those have a single channel conductance of 120pS in CsCl and modest voltage dependency [63]. Cx43, being a gap junction channel-forming protein, has been thought to be associated with the cardiac conduction system. In $Cx43^{-/-}$ mice, till 12.5 dpc, in which Cx40 present abundantly in the ventricle, the ventricular conduction system was found unaffected [5,37]. However, from 17.5 dpc the ventricular conduction was greatly reduced and ventricular arrhythmias were developed in these animals [5,37]. Similar phenomenon was also observed in cultured neonatal myocytes from these mice, and importantly these cells did not show compensatory properties by two other cardiac connexins namely, Cx40 and Cx45 [5,37]. $Cx43^{+/-}$ mice has been reported to be able to grow to the adulthood; however, there were discrepancies regarding the defects in cardiac conduction properties related to the compromised expression of Cx43 protein among relevant studies. Specifically, some reports claimed linear ratiometric reduction in ventricular conduction velocity [5,37,64], whereas other report did not notice any change [65]. Later, conditional knock out of Cx43 in mice showed 40% reduction in ventricular conduction velocity with a 90% lower expression of Cx43 protein [66]. A study with a heart-specific conditional deletion of Cx43, which gradually reduced Cx43 expression in neonatal heart, showed no differences in heart structure and function at birth [67]. The Cx43 level declined to 59% of the control levels by 25 days, but they did not show any anomalies in ventricular conduction. However, after 45 days, the expression levels reached to 18% of the control levels and the heart showed 50% decrease in conduction velocity; later 80% of these mice produced lethal Ventricular tachycardia (VT) [41]. Collectively, these results indicated significant importance of Cx43 for development and maintenance of proper cardiac conduction system in animal.

Connexin 45. Cx45 is one of the principal cardiac connexin protein involved in vertebrate heart development. It is the only connexin that expresses in the early developmental stage of heart, and can be detectable as early as 8.5 dpc in transcript level and by 9.5 dpc in protein level [68]. Cx45 was initially discovered in chick embryo, and 10 folds higher expression in embryonic heart was found compare to the adult heart. The mouse homolog was cloned from embryonic carcinoma cell F9, and was shown to be highly expressed in embryonic tissues [37]. Cx45 was initially believed to be expressed in the working myocardium of mammals. However, it was later clarified that Cx45 is one of the predominant Cx gene expressed in the atrial conduction system constituted of sinus node and AV node [37,69]. Besides, Cx45 gene products were also identified in His bundle and bundle branches. The initial discrepancy related to myocardial expression of Cx45 happened due to the use of antibody that cross-reacts with Cx43 [5]. The expression pattern of Cx45 is similar in human and mice.Cx45 is expressed specially in cardiac conduction system in embryonic and adult human heart [5]. The conserved expression of this gene products in human and mice suggests conserved functionality of these proteins between species. Cx45 knockout mice develop conduction blocks and defects in atrioventricular cushion development, leading toembryonic lethality [68]. Cx45 gene products express in atrioventricular canal, outflow tract, and in the cushion cells during the onset of the appearance of cushion cells. The expression of Cx45 in cardiac endothelium was not observed after the major morphological transformation, and suggested that Cx45 might be involvedin the epithelial to mesenchymal transition (MET) of endothelial cells and early maintenance of the cushion cells [68,70]. Cushion cells are the source of septa andcardiac valves. In addition, the early report has suggested that they can also contribute to the development of cardiomyocytes. The characterization of Cx45 knockout mice also supported this early notion that the cushion cells differentiate into cardiomyocytes, which contributes to the formation of trabecular region. The Ca^{+2}-dependent transcription factor NFATc1 has been shown to be involved in early stage of cardiogenesis. Specifically a rise in intracellular Ca^{+2} dephosphorylates NFATc1 *via* calcineurin, and dephosphorylated NFATc1 then translocate into nucleus to take part in its cooperative gene regulation program [68,71]. NFATc1 localization was restricted into the cytoplasm in $Cx45^{-/-}$ mice,

compare with the nuclear localization in control group. However, NFATc1 deficient mice developed with defects in derivatives of endocardium, and gradual lethality within 13.5 dpc to 17.5 dpc suggested that Cx45 might have other substrates beside NFATc1, which are collectively responsible for specific cardiac phenotype and early lethality of the embryo as early as 10.5 dpc. A cardiac-specific deletion of Cx45 has been achieved in mice, which did not show defects in cushion formation but the mice were embryonic lethal and also die at around 10 dpc due to the failure in pump action [72-75].

Cx45 was found to be increasingly localized to the pacemaker and conduction system of mouse heart during the later stage of embryonic development. Abolished expression of cardiac Cx45 in mice during the embryonic development resulted in severe cardiac defect, leading to embryonic lethal. However, conditional knockout of Cx45 in adult mice did not affect cardiac structure but developed impaired AV nodal conduction [75]. The heart rate was unaffected in such type of adult mice but a slower conduction velocity was reported. Although adult mice with heart specific deficiency of Cx 45 showed slower AV conduction than normal heart, no difference was observed for atrial and ventricular conduction [76]. The conduction system of AV node in adult mouse heart is complex and collectively maintained by three Cxs namely, Cx30.2, Cx40 and Cx45. Mice deficient with Cx30.2 showed accelerated AV conduction whereas Cx40 deficient mice showed decelerated AV conduction [77]. Interestingly, mice deficient in Cx30.2 and Cx40 did not show abnormalities in AV conduction system suggesting that Cx45 might be sufficient for maintaining AV conduction in mouse heart [77]. It was observed that the expression of Cx30.2 was significantly reduced (~80%) in the Cx45 single knockout mice indicating a close cooperativity between these two Cxs in vertebrate heart [77]. Cx30.2 and Cx45 have been shown are able to form heteromeric and heterotypic gap junction channels in culture cells as well as in the cardiac conduction system with a low conductance (~17 pS) [37]. Since these Cxs form slow-conducting (Cx30.2, ~9 pS; Cx45, ~32 pS) homotypic or homomeric channels, and their sensitivity to transjunctional voltage is different (Cx30.2 is lower and Cx45 is higher) therefore it has been proposed that Cx45 has a stabilizing role for Cx30.2 channel [5,37,78]. Further, it has been proposed that Cx30.2 and Cx45 heteromeric/heterotypic channels might be critical for preventing excessive quick opening of conduction channels in AV node initiated by higher atrial voltage [78]. This notion was supported by the fact that Cx45 expression appears in early stage of heart development when faster conduction might be important whereas Cx30.2 express in comparatively later stage to support the requirement of comparatively slower conduction in matured heart.

Connexin 40. Cx40 has been reported expressed first in the atrial compartments and later in the ventricles at 11 dpc during embryonic development of mouse heart [79]. The expression becomes to be progressively restricted to the conduction system on and after 14 dpc; however, the expression of Cx40 in working atrial myocardium remains evident [45,80]. Unlike Cx43, Cx40 does not express in the muscular regions of interventricular septum of mouse heart. Cx40 knockout mice revealed that Cx40 is critically important for the generation of the mature apex to base activation in heart [81]. Studies with Cx40 knockout mice further revealed that Cx40 is necessary for proper development of functional heart [80-82]. Cx40 knockout mice developed with high incidence of different forms of CHDs, including VSD, TOF, DORV, and defects in endocardial cushion and aortic arch. However, considering the fact that such kind of defects were observed in 30% of Cx40 homozygous knockout mice, it was suggested that the defects might be linked with the down-regulationof Cx40 in the neural crest, which likely indirectly affectsproper heart development [83]. Hand2 has been recently shown to be expressed in neural crest cells, and can affect heart development that produced DORV or VSD phenotype. The same study further revealed that Cx40 expression in neural crest was reduced in Hand2 knockout mice, suggesting coordinated activities of Cx40 and Hand2 in heart development [84].

Studies in human revealed that Morbus Fallot is correlated with above-average incidence with copy number variants in Cx40 gene [5]. It was further showed that Cx40 expression in the RVOT, which is malformed in Fallot's Tetralogy,is elevated in TOF patients [5]. It has been previously

shown that mutations in the C-terminal domain of Cx43 are directly associated with many pathophysiological conditions related to CHDs [5]. A recent study in human indeed revealed significant information regarding the relationship between the integrity of the C-termial domain of Cx40 and heart defects [85]. Guida et al. analyzed more than 150 patients with non-syndromic Fallot and found that~1% of the patients hada heterozygous mutation in Cx40 in which the Pro residue at amino acid position 265 is converted to Ser (P256S) [85]. P256S is a bona fide binding site for src and has been shown to be important for Cx biochemistry. Specifically, P256S led to reduced gap junctional coupling of Cx40, and was shown to be critically important for proper heart development in zebrafish model [85]. Such a mutation was not observed in healthy human samples, thus indicating the relevance of the proper functionality of Cx40 in CHDs.

Cx40 forms gap junction channels with a large single channel conductance (~150 pS), which is even larger than that of Cx43 channel (~120 pS, in CsCl) [86]. Atrial myocytes coexpress both of these gap junction channel forming proteins. It has been shown thatCx40 and Cx43 can form both heteromeric and heterotypic gap junction channels in mammalian cells, including adult atrial myocytes [87-89]. Cx40 and Cx43 contributed equally to the total gap junctional conductance in cultured neonatal mouse myocytes [88-90]. Ratiometric analysis further revealed that the ratio of Cx40 and Cx43 is a key factor for the propagation of conduction velocity in atrial myocytes. Precisely, Cx43 positively and Cx40 negatively influence the conduction velocity in such experimental system. The incidence of heart malformations is worsened when $Cx40^{-/-}$ and either $Cx43^{+/-}$ or $Cx45^{+/-}$ are combined in same mice. The $Cx40^{-/-}$ – $Cx43^{+/-}$ mice died in the embryonic developmental stage,whereas $Cx40^{-/-}$ – $Cx45^{+/-}$ mice developed with defects in AV conduction. Moreover, $Cx40^{-/-}$ – $Cx43^{-/-}$ mice died earlier at around 12 dpc with an abnormal rotation of the ventricles [5,37,90].

The Cx40 gene has been suggested to be regulated by three major transcription factors related with cardiogenic programs, *viz.*, GATA4, Nkx2.5, and Tbx5. GATA4 Nkx2.5, and Tbx5 could interacts specifically with the elements existing in the minimal promoter region (within 150 nucleotides) of the Cx40 gene [91]. GATA4 and Nkx2.5 were found to upregulateto Cx40 gene expression [91]. This observation was supported by the findings that Cx40 expression was significantly depleted in Nkx2.5 knockout mice. On the contrary, Tbx5 was shown to be negatively regulate Cx40 expression [92,93]; however, the precise control of Tbx5 on Cx40 expression might be multifactorial. Two other homologs of Tbx5, *viz.*, Tbx2 and Tbx3 were also to be found repressive for Cx40 expression [94,95]. As already mentioned earlier,the transcription factor Hand2 also might directly regulate Cx40 expression at least in the embryonic developmental stage.

Connexin 31.9. Cx30.2 is the mouse ortholog of human CX31.9 [96], and was found to be expressed in sinoatrial node, atrioventricular node and His bundle in mouse heart. Cx30.2 forms weakly voltage-dependent and small conductance (~9 pS) channels [97]. It can also form heterotypic and heteromeric gap junction channels in mammalian cells with the principal cardiac connexins, *viz.*, Cx40, Cx43, and Cx45 [98]. In mouse heart Cx30.2 is colocalized with Cx45 in SA and AV nodes but not with Cx40 and Cx43 [97]. A deficiency in Cx30.2 causes accelerated conduction in AV node. Cx30.2 and Cx40 dual knockout mice did not show any abnormalities in AV conduction system, suggesting that the perfect balance of Cx30.2 and Cx40 is important for proper AV conduction in mouse heart [77]. Cx30.2 expression is regulated by GATA4 and Tbx5 during the developmental stage [14]. Further it was found that inhibition of Notch signaling pathway depleted Cx30.2-expressing cells, accelerated conduction of AV node, and developed hypoplastic AV node in mouse model. Reportable that CX31.9does not express in the conduction system or myocardium signifying critical difference in functions of this Cx protein between two species [99]. It has been suggested that Cx30.2 in small mouse heart might be important to allow high activation frequencies and optimized A – V timing for efficient pump action [99].

The role of this Cx protein in heart development was recently discovered by our group using zebrafish model. By using synteny analysis and phylogenetic analysis we have identified that Cx36.7 (zfCx36.7) is the zebrafish homolog of human CX31.9 [11]. Through genetic screening of randomly

N-ethyl *N*-nitrosourea (ENU)-mutagenized pool of zebrafish, we have identified a mutant with specific heart phenotype, futka (*ftk*). The mutant develops with a malformed heart that beats irregularly and showed severe regurgitation defects. The heart wall of *ftk* remains thinner than the wild-type heart, but atrioventricular valve formation was found to be normal despite showing severe regurgitation defects. The trabecular development in *ftk* heart was also seriously impaired. The *ftk* heart punctures at the later stage of embryonic development, and the animal die from blood loss due to bleeding into the pericardial cavity. Ultramicroscopic analyses revealed that the myofibrils are distributed anisotropically in *ftk* heart, compared with the organized unidirectional distribution of these fibers in normal heart [11], which is a fundamental requirement for directional co-ordinated heart beating.

The phenotypes described above can be attributable to this myofibrillar disarrangement. For example, (1) irregular rhythm of the mutant heart may result from the aberrant arrangement of myofibrils that prevent coordinated directional movement of cardiomyocytes. Similarly, it also impeded the kissing of the atrioventricular junction-cushion to work as an early functional valve, thus allowing severe regurgitation. (2) The leakage of red blood cells across the cardiac chambers may be due to the weakness of the chamber wall consisting of random arrangement of myofibrils and stretch stress by non-coordinated beating. For the dilated heart of the *ftk*mutant, Frank-Starling law also diabolically facilitates the damage by exerting extra pumping force [100,101]. (3) Although the reason for the defect in trabeculae formation is not clear, the defect may suggest that parallel arrangement of myofibrils is necessary for the trabecular development. The expression of Nkx2.5 was down-regulated in the *ftk* heart despite the normal expression of two other essential cardiogenic transcription factors GATA4 and Tbx5, suggesting a common mechanism for the occurrence of *ftk* phenotype and congenital heart disease (CHD) [11]. Our study revealed that zfCx36.7 is the responsible gene for this severe heart defect. Particularly, a missense point mutation in the N-terminal region of zfCx36.7 replaced a conserved acidic residue Asp at amino acid position 12 to Val (D12V). We found that zfCx36.7 can work as a functional hemichannel at the plasma membrane but D12VzfCx36.7cannot reach to the plasma membrane (Fig. 3). In addition, our recent work indicated that this D12V mutation abolished the interaction of zfCx36.7 with ZO-1 (unpublished data), which is an important regulator for the plasma membrane targeting of a candidate protein.

Generally Cx proteins are involved in nodal signaling in heart [102,103]. However zfCx36.7 is not related with such type of functional relevance of working heart but exclusively related with the development of heart as suggested by cardiac tissue specific expression of the transcript only in the early stage of heart development [11]. In fact, we found that zfCx36.7 is most likely the earliest marker for cardiogenic cells (as early as 50% epiboly stage), and therefore we proposed it as early cardiac connexin (ECx). The absence of human homolog CX31.9 in adult heart tissue is completely in accordance with zfCx36.7 expression data. However, there is no information whether CX31.9 is spatiotemporally express in human embryonic heart during the early stage of development similar like zebrafish model and warrant detail analysis of such prospect.

The simplest possibility of myofibrillar anisotropy in the early cardiomyocytes of *ftk* could be the secondary effect of irregular contraction-expansion cycle due to the non-functionality of the mutated zfCx36.7. Yet, zf Cx36.7 seems not to be related to the nodal function because the message is highly expressed during the early stages of heart formation in the non-pulsating differentiating cardiomyocytes, which decline after the heart beating (after 24 hour post fertilization, hpf) – the period when the demand of nodal signalling is high [11]. The zf Cx36.7expression is not detectable at all after 72 hpf, when the heart is fully functional. Taken together, our observations lead us to propose that the connexin zf36.7 forms a hemichannel on the plasma membrane of developing cardiomyocytes, and allows some small molecule/s to get into these cells, which may directly or indirectly activates Nkx2.5 expression. Nkx2.5 then activates its substrate genes to execute the proper heart development program (Fig. 3). This is the first report to show that Cx protein not only exclusive for nodal signaling in heart but also have a direct role in the cardiogenic program.

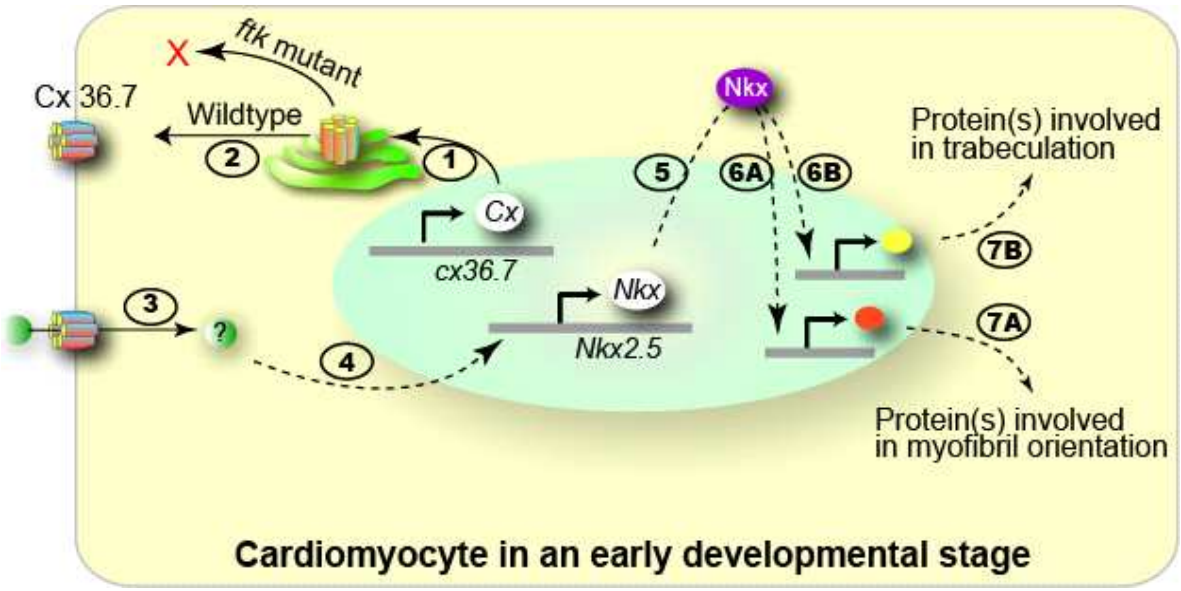

Fig. 3. Early cardiac connexin Cx36.7 and its role in heart development. In the developing cardiomyocyte, zfCx36.7 forms a hexameric connexon on the plasma membrane. However, mutant zfCx36.7 cannot reach to the plasma membrane and are responsible for *ftk* phenotype. Normal zfCx36.7 allows some small molecule/s to get into the cell and establishing the Nkx2.5 expression. Nkx2.5 in turn activates multiple genes related to heart development. The sequences of the activities are shown with serial numbers from 1 - 7.

Other Cardiac Connexins. Cx30, Cx37, and Cx46 are other connexin proteins expressed in vertebrate heart [5,37]. Cx30 was found to be expressed in SA node of mouse heart. It forms channels with a large single channel conductance (179 pS) [104]. Mice with depleted expression of Cx30 showed higher heart rate over the normal mouse heart and beat irregularly, which suggested that Cx30 is involved in intrinsic pacemaker frequency [105]. Cx37 is expressed in embryonic ventricular myocardium, endocardial lining of both chambers (atrium and ventricle), mesenchyme of conotruncal ridges, atrioventricular cushions, endothelial cells in blood vessels, and in small level in ventricular trabeculae. Cx37 can form large conductance channels that are moderately voltage sensitive [106,107]. Cx37 homozygous knockout mice indicated that this protein might be critical for the proper development of venous and lymphatic valves [108]. Cx37 and Cx40 dual knockout mice showed higher occurrence of atrial and ventricular septal defects [109]. Cx46 also forms a larger conductance channel (~140 pS) [110], and mainly expressed in the lens of eye. However, the expression of Cx46 was reported in canine heart [69]. Minor level of expression of Cx46 was detected in between some atrial and ventricular myocytes [43]. Cx46 expression was reported in the atrium, interventricular septum, AV canal, and ventricular subendocardiumin newly born mice. Cx46 can form functional hemichannel but their role in heart development is unknown [39].

Recovery of CHD by Exploiting Connexin

The roles and regulations of Cxs in cardiac development and proper functions have been described in the preceding sections. The following section will focus on possible biotechnological approaches to recover CHDs in connection with Cx gene products. Cx proteins can be regulated by post translational modifications such as phosphorylation, nitrosylation, ubiquitination, lipidation, hydroxylation, methylation etc. Such kinds of post-translational modifications critically regulate the expression, channel formation, trafficking, channel gating, internalization, recycling and degradation of Cx proteins, and are related to the pathophysiological conditions of CHDs [18,19,22,26,27,43,55,111-118]. Several types of biotechnological approaches can be attempted to recover Cxs-mediated CHDs, 1) including pharmacological approach by applying small chemicals, 2) peptidomimetic approach, and 3) stem cell technology and regenerative medicine approach.

Small Molecule Approach. Various types of small molecules have been applied to regulate Cx proteins and their channel function [119]. These molecules cover a wide range of functional pharmacological agents, including polyamines, tetraarylammonium ions, cyclodextrins, cisplatin, fatty acid amides (e.g.,oleamide), triarylmethanes, antimalarial agents (e.g.,quinine and mefloquine), fenamates, 2 aminophenoxyborate, glycyrrhetinic acid, and volatile anesthetics (e.g., halothane and ethrane). Most of the reported inhibitors simultaneously inhibit gap junction channel and hemichannel function [119]. Without minor exception, for example, carbenoxolone cannot abolish Cx43 junctional coupling even at high dose, most of these molecules can completely inhibit channel function of Cx protein. However, neither of these molecule has selective regulatory activities on specific Cx protein with only few exception, for example, antimalarial drug quinine and mefloquine show better selectivity for Cx36 and Cx50 over Cx26, Cx32 and Cx43 [120]. Since a single cell can express multiple Cx homologs therefore non-selective inhibition or activation of Cx proteins in same cell or tissue could develop unexpected complications. Currently there are no small molecule inhibitors that can bind with any Cx protein at a low molarity. Most likely the chemical molecules, which are tested, are too small (compared to the larger size of the Cx conduit), and therefore these molecules cannot directly block the Cx channel pore. Carbenoxolone-mediated inhibition of Cx channel function reached steady state after 10-15 min, suggesting that an indirect mechanism controlling protein turnover or trafficking of Cx protein might be affected [17].

Some polyamines, such as spermine and spermidine, preferentially inhibit Cxs; Cx40 is highly sensitive and Cx43 is insensitive. Mutagenesis experiments revealed that the inhibitory effects of polyamines are exerted *via* N-terminal region of Cx protein [119]. Particularly, the inhibitory property of spermine is abolished by substitutions of two acidic residues in N-terminal intracellular domain of Cx40 (E9 and E13) with the corresponding positively charged residues of Cx43 (K9 and K13) [121]. This suggests that Cxs with these acidic residues in the N terminal intracellular domain can be satisfactorily inhibited by polyamines. However, polyamines can interact with many different kinds of cellular substrates like nucleic acid, phospholipids, and acidic proteins, and influence gene expression, signaling pathways, ion channels, and enzyme activities related to cellular growth and animal development. Therefore, using such kind of Cx inhibitors is not an attractive choice, if the objective is to use them for *in vivo* experiments.

Peptidomimetic Approach. Peptidomimetic represents a short string of amino acids that mimic a natural peptide sequence. Cx petidomimetic means such type of short amino acid oligomers that mirror a peptide sequence of a Cx protein. From a biotechnology point of view, designing and synthesizing such kind of mimetic peptides are easily achievable. Biomimetic peptides for Cx protein was first introduced in 1994 by Dahl et al [122]. The study used *Xenopus* oocytes with ectopic expression of Cx32, and analyzed the efficiency of dodecapeptide mimetic sequences to disrupt gap junction communication formed by Cx32. In 1995, Warner et al. screened several candidate sequences using the chic myocyte bioassay system to identify functionally relevant Cx mimetic peptides [123]. The study considered multiple short sequences of amino acid spanning the whole range of two extracellular loops (E1 and E2) as candidate Cx-mimetic peptides by evaluating

the capacity of tampering the channel property. The data were evaluated by using intracellular peptides with comparable lengths as negative control. Consequently, two mimetic peptides, *viz.*, Gap26 and Gap27, were successfully established corresponding to the first and second extracellular loops of Cx43, respectively [124]. The sequences for the mimetic peptides were selected from conserved sequences of the relevant domains, which are VYCD for Gap26 and SRPTEK for Gap27. Gap26 and Gap27 peptides function in a Cx-specific manner, and selectively applied for inhibition of Cx37, Cx40 and Cx43 [17,125]. These two Cx43 mimetic peptides can selectively inhibit gap junctional intercellular dye transfer, electrical coupling, and synchronized Ca^{2+} wave in smooth muscle cells [126]. An altered Gap27 peptide was generated by incorporating Cx40-mimicking sequence, which was also successfully applied for selective regulation of Cx channels in vascular tissues and other tissues where both Cx40 and Cx43 are expressed.

The principal activity of Gap26 and Gap27 has been suggested to be directed to the hemichannel function of Cx protein [17,125]. It has been reported that mimetic peptide-mediated inhibition of Cx function is reversible, which is advantageous for controlled regulation of Cx channel for specific period of time. In HeLa cells stably transfected with Cx43 both of these mimetic peptides inhibited unitary currents within short time, indicating selective blockage of Cx43 hemichannel function [125]. A similar observation was reported using pig ventricular myocytes that express endogenous Cx43. However, Gap27 mimetic peptide-mediated inhibition of Cx channel function reached steady state after ~60 min of transfection suggesting that an indirect mechanism like protein trafficking or turnover might be affected [127,128].

Many other Cx mimetic peptides have been designed from the intracellular loop and C-terminal region. To reach their sites of action they need to cross the cell membrane, which can be achieved by adding additional cell-penetrating membrane translocation motif. Suitable nanocarriers also can be used for effective delivery of such kind of mimetic peptides to target cells. Our lab also developed several highly efficient gene delivery methods into mammalian cells, including highly safe Calcium-apatite nanoparticle carrier [129-133]. However, mimetic peptide with net positive charge, for example, with several Lys residues, can penetrate plasma membrane without any carrier. Considering that mimetic peptides are very small with molecular weight less than 1 kD, it is most likely that they can exploit Cx hemichannel to reach their targets without the need of direct penetration of plasma membrane.

A nonmimetic hexapeptide ZP123 showed higher degree of phosphorylation to Cx protein and enhanced gap junctional coupling [134]. It also prevented VT in an animal model suffering from myocardial ischemia, which is a promising finding for advancing biomimetic approach for the treatment of Cx-mediated CHDs. ZP123 has been shown to enhance ATP release in a ischemic cardiac myocyte model [134-136]. However, Gap26 blocked ATP release in the same system suggesting their selective roles on Cx. Gap26 has been further shown to provide protection to myocardial ischemic model from hypoxic stress by binding with Cx43 hemichannel and blocking its function [17,125]. A close homolog of Gap27 was successfully applied to reduce vascular leak in an atrial ischemia model, which was most likely involved in enhanced expression of Cx43 hemichannel in relevant tissue. Hennan at al., have recently introduced another Cx mimetic, Gap 134 dipeptide [137], as an effective antiarrhythmic agent with potential to reduce ischemia/reperfusion injury in a canine model.

A recent study by Beyer et al., has introduced a new Cx mimetic peptide, interfering NT (iNT), with the amino acid sequence corresponding to the conserved N-terminal domain of Cx spanning residue 9 – 13 [138]. A Cx43 iNT peptide with the sequence Ac-KLLDK-NH2 selectively inhibited the coupling of Cx40 in a transjunctional voltage-dependent manner. Importantly, it did not affect the channel function of close Cx homologs, *viz.*, Cx37, Cx45, Cx46, and Cx50. The study has further shown thatthe voltage dependent blockage of the Cx43 iNT peptide was counteracted by using Cx40 iNT with the amino acid sequence Ac-EFLEE-OH but not Cx50 iNT with the amino acid sequence Ac-EEVNE-OH [138]. Indeed, Cx40 iNT could not inhibit Cx43 channel function at all. This study clearly suggests that the Cx43 iNT peptide may selectively antagonize anomalous function of mutant

Cx40 hemichannel or gap junctional channel associated with atrial fibrillation, and may be applied for recovery of relevant CHDs.

The two highly conserved extracellular loops of Cx protein was found poorly immunogenic and therefore generating antibodies for these two domains was problematic. Moreover, it was argued that the larger sizes of antibody molecules are not suitable to apply them for the regulation of Cx protein function in a tightly formed tissue, like heart tissue. Biomimetic peptides, on the other hand, cannot only reach to these cells but also can penetrate through the intercellular connection or can directly enter through Cx-hemichannel to reach targeted Cx protein. Extracellular domain targeting mimetic peptides like Gap26 and Gap27 can even directly reach to their target without the need of special delivery system. The better selectivity of mimetic peptides over small molecule regulators is also advantageous for applying them to regulate specific Cx-channel to overcome relevant defects in CHDs.

Stem Cell Technology and Regenerative Medicine Approach. Heart transplantation is an established procedure to treat defective heart but is highly restricted due to the unavailability of a donor organ [139]. Stem cell technology and regenerative medicine could therefore be a suitable approach to treat CHD patients. Stem cell is the starting materials for regenerative medicine and tissue engineering. Stem cells are scientifically categorized into four major types – (1) embryonic stem cells (ESC), (2) adult stem cells (ASC), for example mesenchymal stem cells (MSC), (3) induced pluripotent stem cells (iPSC), and (4) pathological stem cells, for example cancer stem cells (CSC) [140]. Among them, ESC and iPSC are being recognized as true pluripotent stem cells. Pluripotent stem cells have the capacity for unlimited self-renewal and differentiation into all the approximately 200 specialized cell types of the body, and therefore they have been considered to be the most favorable starting cells [141]. Earlier studies have successfully produced cardiomyocytes from ESC [142]. However, ESC-derived cardiomyocytes are not suitable for transplantation in human because of immunological consequences and graft rejection. In 2006, Yamanaka group discovered the art of making iPSC from mouse somatic cells [143], and human iPSC generation follows soon after [144]. The introduction of iPSC has revolutionized the field of stem cell technology and regenerative medicine field, and now there are several hundred iPSC lines available to the scientific communities. Despite having been some concerns that ESC and iPSC are not identical, however, they are morphologically same and has been shown can be differentiated to all the three germ layers including cardiomyocytes. Recent studies showed that iPSC can be efficiently differentiated to cardiomyocytes by using cocktails of small molecules and growth factors [142,145,146]. Our lab also recently have shown that by using surface immobilized factors, *viz.*, insulin-like growth factor binding protein 4 (IGFBP4) fused with elastin-like polypeptides significantly enhanced cardiomyocytes differentiation from mouse ESC [147]. We further found that niche in culture system can accelerate cardiomyocytes differentiation from mouse ESC and their maturation (unpublished). The differentiated cardiomyocytes then need to be enriched or purified from unwanted cellular contamination like undifferentiated stem cells to avoid risk of tumor formation; current protocols can enrich differentiated cardiomyocytes at high purity [148,149]. The ESC- or iPSC-derived cardiomyocytes resemble with the cardiomyocytes of developing mammalian hearts, which matures at a later stage. Recent study reported that cardiomyocytes derived from human pluripotent stem cells successfully displayed electophysiological coupling and suppressed arrhythmia in guinea pig model [150]. This observation indicates that cell transplantation therapy can be an effective treatment option for recovering CHDs.

The early protocols used retroviral or lentiviral genetic integration methodologies to generate iPSC [143,144]. The generated iPSC from such protocols harbored transgenic elements from the viral vector, which randomly integrate to the genome of the experimental cells and is a major safety concern for application in regenerative medicine. This issue has been successfully overcome recently by using non-integrating genetic system like plasmids, mRNAs, proteins, removable transposons etc. Therefore generation of iPSC with corrected expression of a malfunctional gene or patient-specific iPSC, which could later be differentiated into cardiomyocytes using existing protocols and can be

transplanted into the affected heart, could be an attractive strategy for the treatment of CHD patient [142]. Our study showed that wild-type zfCx36.7 was able to recover the trafficking defect of mutant zfCx36.7 in mammalian cells (unpublished data). Not only that, co-expression of normal zfCx36.7 was able to restore hemichannel function of the mutant Cx in a dose dependent manner is a clear indication that such type of engineered iPSC with corrected expression of affected Cx gene might be a justified approach to treat Cx-related CHDs. Designer gap junction protein model has been recently proposed [151], which are resistant to pathologic remodeling. It would be interesting to introduce such type of engineered Cx gene into iPSC for evaluating the prospect of using them for the treatment of CHD. Gene therapy technology also has been suggested for the recovery of Cx-mediated craniofacial regeneration problem [152], indicating that CHD-related defects for Cx also might be recovered by this approach.

Cx expression in cardiac tissues has been reported to be controlled by thyroid hormone. Thyroid hormone treatment of cultured neonatal cardiomyocytes significantly upregulated Cx43 expression and enhanced gap junction channel formation [153]. It was further reported that thyroid hormone can induce expression of Cx40 transcript in mouse atria in a receptor-dependent manner [154]. Recently intramyocardial transplantation of genetically modified MSCs (to express angiogenic factors, for example, VEGF, bFGF or IGF-1) has been shown associated with increased expression of contractile proteins and improved left ventricular function in ischemic myocardium [155,156]. Similarly, MSCs genetically modified to express thyroid hormone can be transplanted into defective heart that can function as a local source of this hormone to the neighboring heart cells to regulate expression of relevant Cxs.

GATA4, Nkx2.5, and Tbx5 are the major transcription factors related to vertebrate heart development. Nkx2.5 knockout mice demonstrated that the Nkx2.5 transcription factor is critical for the expression of three important cardiac Cxs, *viz.*, Cx40, Cx43, and Cx45. Our study with zebrafish mutant *ftk* revealed that zfCx36.7/ECx is the upstream factor of Nkx2.5, suggesting a close connection between the regulatory systems of cardiac Cxs and the cardiac transcription factors [11]. The fact that Hand2 knockout mice and Tbx5 heterozygous mice developed with CHD-like phenotype, and Cx40 expression has been significantly reduced in these mice further supporting this notion. Therefore genetically engineered iPSC with corrected functionality of affected Cx protein/s obtained from CHD patients indeed hold strong prospect for relevant treatments.

Conclusion

Piles of studies have established that malfunction or altered expression of Cxs are associated with several types of CHDs [5,37,116,157,158]. Multivariate roles of cardiac connexins, expression of multiple Cx homolog in one cell and heterotypic as well as heteromeric channel formation made them very complex system for drug discovery target. The intracellular and intercellular localization of the same Cx protein, as exemplified by the mitochondrial and cell-surface localization of Cx43 hemichannel, have provided further twist to the complexities. Since proper combinations and distributions of Cxs in vertebrate heart are essential for maintaining appropriate and localized electrical coupling therefore while non-selective inhibition of gap junction by small molecule can be beneficial for some region but at the same time it might be very detrimental for other region of heart. Although several studies indicated that selective regulation of Cx is not impossible but it remains a huge challenge for developing and establishing highly selective molecule for application in clinical testing. Nonmimetic hexapeptide ZP 123 (rotigaptide) showed promise for the treatment of VT in myocardial ischemia canine model; however, rotigaptide did not produce conclusive results in clinical trial and therefore was discontinued from further development [134-136]. It is essential to develop effective high throughput-screening technology to identify new selective regulator for Cxs. Currently, there are no such system that can conclusively provide suitable read-out to detect candidate molecules for further evaluation [119]. Existing technologies, for example fluorescence microscopy- or luminescence -based analysis, are not suitable for direct-acting candidate molecules.

Since, for this kind of technology the candidate molecules need to add to the system before addition of cells therefore it is not clear whether the read-outs are for direct or indirect effect. Such kind of technology is also not suitable to study intracellular hemichannel function. Cardiac Cxs have been shown can be regulated by autocrine/paracrine factors [159], and therefore transplanting engineered cells to secret local stimulant for the regulation of Cx proteins in heart tissue could be an promising approach to recover defects in cardiac Cxs and thereby associated CHDs. However, it is necessary to evaluate detail physiological complicacies relevant to such type of technology. Continuous efforts from scientists have been advancing stem cell technology for application in the cardiac regenerative medicine filed. Despite significant advancement in the related filed the issues related for efficient and quicker transformation of somatic cell to iPSC and differentiation to cardiomyocytes for clinical application need to be perfected. Direct conversion of somatic cells to cardiomyocytes could be a good strategy [142,160], where targeted Cx gene or Cx-regulatory gene can be inserted, and cardiomyocytes with corrected Cx expression can be achieved within a faster time frame. However, detail evaluation of the proposed technologies *in vivo* mammalian model, as well as *in vitro* human system shall provide clear understanding of such approach. Although we have proposed some possible biotechnological approaches for regulating Cx gene products in heart tissue related to CHDs, nevertheless similar approaches can be applied for global or local regulation of abnormalities of Cx gene products associated with other pathophysiological conditions.

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Nano-TiO_2-enriched biocompatible polymeric powder coatings: Adhesion, thermal and biological characterizations

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Abstract. The success of orthopedic and dental implants largely depends on their biocompatibility with the surrounding body environment and the biocompatibility depends on the physical, chemical, mechanical, topographical and biological properties of the implant materials chosen. Since the last few decades, titanium and its alloys have been among the most widely used ones due to their superior biocompatibility and mechanical properties; however, pure titanium needs to be pre and/or post treated chemically or physically to maintain appropriate textures and surface roughness. In the present study, TiO_2 nanoparticles incorporated polymeric powder coatings consisting of smooth and micro-nano scale roughness were developed that exhibited biocompatibility towards Human Embryonic Palatial Mesenchymal (HEPM) Cells. In addition, an experimental set up was designed and executed to evaluate the adhesion/ bond strength of the coating and to measure the load bearing capacity that the coatings can withstand before being detached from the substrate. Coating's topographical features were analyzed by using Scanning Electron Microscopy (SEM). Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were performed to evaluate the thermal stability of the coating materials.

Introduction

Bone is a natural composite material that mostly comprises of collagen fibers and apatite. As people grow older with age, the bones become less dense and the strength of these bones also decreases, hence becoming more susceptible to fracture, bone defects and bone resorption caused by infection. Bone replacement materials provide appropriate solutions when a section of bone is missing or the gap needs to be filled.

In the past decades, synthetic materials have become popular as bone replacement materials. A number of metals and ceramics have widely been considered for this purpose, despite none of them provides a perfect combination of physical, mechanical, chemical and biological properties necessary to be compatible into human body. However, polymer composite biomaterials provide a better alternative choice because of their biocompatibility and corrosion resistance [1-3]. Composite materials have the advantage of high specific modulus and strength to weight ratio too, which makes them to be a prospective material in tissue replacement and bone regeneration. A wide range of nanocomposite materials are being developed nowadays in order to design and synthesize novel biomaterials with specific properties, such as high biodegradability or bioactivity [3, 6-9]. Moreover, since polymer-inorganic hybrid composite materials have the advantages of having both the organic polymer and inorganic component, they have been studied extensively for their biomedical applications [1-10].

However, yet titanium remains to be the center of interest of to many laboratories worldwide due to their unique combination of mechanical and biological properties as well as corrosion resistance. However, Ti implants are reported to be linked with local inflammation ranging from skin rashes to complete implant failure [11]. Moreover, the osseointegration of orthopedic and dental implants is dependent on the interactions that occur between their surfaces and the surrounding tissues, which in turn depends on implant surface chemistry, roughness [12-14], and topography [15-17]. Titanium implant surface modifications have involved various polishing, grit blasting, machining, microfabrication, as well as acid and chemical etching techniques [14, 18-22].

In particular, surface roughness and topography that are within the nanoscale have been reported to enhance the cellular response [4-6, 23-28]. Nano-rough topographies may actually mimic

nanostructured proteins and crystals that are normally present within tissues [29]. As a consequence, nanoscience provides us with a means to improve materials' bulk and surface properties by manufacturing nanomaterials. Structures with nanometer diameters have attracted great attention due to their promising applications in electrical, optical, magnetic, and medical fields [30]. For materials requiring specific features to adhere to its properties, nanostructure elaboration has given the answers, which applies to titanium as well. Anodic oxidation is not only inexpensive but a convenient electrochemical method, efficiently used in TiO_2 nanotubes manufacturing which results in the formation of attached nanotubular or nanoporous structures on the substrate surface [30]. When grown from pure Ti foil, highly ordered, vertically oriented TiO_2 nanotubes form whose diameters, thickness and length can be fine-tuned according to need. Furthermore, these nanotubes, as proven by previous studies, can significantly improve osteoblast attachment, function, and proliferation while exhibiting very low immunogenicity, eliciting low levels of monocyte activation and cytokine secretion [31].

As a result, optimization of the surface properties of titanium implants can facilitate the adhesion of bone-forming cells and thereby may promote osseointegration [30]. As mentioned earlier, rough surfaces exhibit increased activity and production of different enzymes, collagen, prostaglandin E2, and transforming growth factor. The surface structure of materials influences the response of the cells and the development of the inter-connective tissue which forms in between the implant and the host body. It is predicted that osteoblasts form more calcium on titania nanotubes substrate than on pure titanium, and this is because bone cells are accustomed to a nanoscale tubular environment rather than to a micro-scale environment. The degree of adhesion is a property of the interphase created between the surface of the implant and the bone cells, playing a key role for the integration of the implant in the human body. Surface properties, such as surface roughness, surface chemistry, and topography of the implants [12-17], influence the initial cell response at the cell–material interphase, ultimately affecting the rate and quality of the newly formed tissue [30].

The dependency of cellular behaviors on nanoscale details [4-6, 23-28] is a reflection of the fact that tissue is a complex and highly organized structure which integrates details at every scale to perform several functions. Some recent studies have focused on the creation of submicron features and nano-rough surfaces on the polymers alone. For example, Chun et al. etched poly (ether) urethane (PU) and PLGA surfaces with nitric acid and sodium hydroxide, respectively, to create nano-rough surfaces. They found that there were higher urothelial cell densities on the etched PLGA surfaces [32]. Similarly, Ranjan et al. fabricated arrays of nano-grooves and alternating micro-nano roughness on poly(dimethylsiloxane) (PDMS) films for cardiovascular applications [9]. They found enhanced endothelial cell adhesion and elongation on the modified surfaces.

In addition to that, we also have studied and developed polymeric powder coatings that possess submicron features and that are capable of supporting favorable cellular responses [33-36]. We used an ultrafine powder coating process with polyester resins, pigment grade micron-sized TiO_2, nano-TiO_2, and polytetrafluoroethylene (PTFE) to create thin, even, and continuous coatings on titanium substrates [37, 38]. These coatings had complex surface topographies and high levels of surface roughness in the range of 40–300 nm, and the developed coatings supported human mesenchymal cell attachment, growth and osteogenic differentiation at levels that were comparable with that on commercially pure titanium (cpTi), which was due to presence of nanoroughness. However, coatings' adhesion (to the substrate) and thermal properties might also have affected the cellular response since some of our formulations contained PTFE. Therefore, apart from their surface features and biological properties, the coatings' adhesion [39-42] and thermal properties [43-46] are also crucial when they are aimed to be implanted inside human body with greater success.

Therefore, in the current study the objectives are to develop polyester-TiO_2 nanobiocomposite coatings by using ultrafine powder coating process followed by their characterization in terms of physical, thermal and biological properties. A lab-scale coating adhesion tester is designed and fabricated to measure the maximum normal force the coatings can tolerate before being delaminated from its substrate. The fabricated composite coatings will also be characterized by using Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA), described elsewhere [43-49] in order to determine whether the PTFE content changes the thermal decomposition properties of the coatings.

Materials and Methods

Preparation of the Coatings. Polymeric powder coatings (PPCs) were prepared by ultrafine powder coating technology [37-39]. Avalanche white polyester powders (Links Coating, London, Canada), filler, pigment (i.e., micron-sized TiO_2), flow (i.e., P10), degassing (i.e., benzoin), and curing agents

were mixed, processed and extruded as shown in Fig. 1 and mentioned in [33-36]. Upon being cooled, the hardened chips formed after extrusion were then ground into ultrafine powder particles of size range between 15 and 20 μm. The fine powders were then mixed with varying concentrations (0-3 wt.%) of nano-sized PTFE (Dupont, Canada) and titanium dioxide (0.5 wt.% nTiO2; Degussa, USA) nanoparticles through bry-blending followed by high-shear mixing of about 30 seconds. The oversized particles were removed by passing through a 32-μm sieve [39] and the ultrafine powders were fed to a corona discharge spray gun through a hopper. A 15-20 kV voltage was applied to ionize the particles as they were applied/sprayed onto the grounded metal sheets. The coated surfaces were then cured (200 °C for 10 min) and cut into small disks of 13.5 mm diameter.

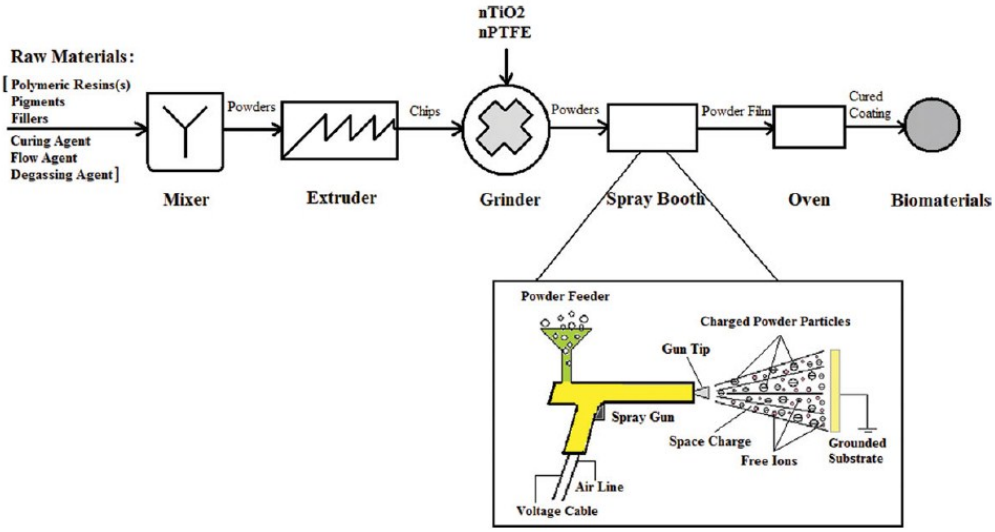

Fig. 1. Powder coating process [35].

Evaluation of Adhesion Strength

Method 1: The adhesion of the PPC to the substrate was examined by an Elcometer 107 Cross Hutch Cutter (Elcometer Ltd., Windsor, Canada). The coated surfaces were cut down to the substrate with a blade ($11\times1.5mm^2$), as recommended by ASTM D3359. Several perpendicular cuts were made to create a grid of small squares. The lattice was brushed to remove debris and was covered with an adhesive tape (ASTM standard) followed by firming it with a pencil eraser. The adhesive tape was then withdrawn by a single smooth pull, and the remaining grid squares were counted and compared to determine coatings' retention to the substrate. The lattice was then assessed for adhesion by using the ASTM D3359 standards.

Method 2: The adhesion strength between the coating and the substrate was evaluated through an especially designed and fabricated loading system shown in Fig. 2. Two aluminum rods of 10 mm diameter and 60 mm length were machined by turning machine. One of the sides of each rod was polished using fine sand paper. A hole of 5 mm diameter was drilled at the other end at 5 mm distance from the end of each rod to hang them in the two jaws of the universal testing machine through strong metallic wires. The polished face of each rod was brushed with a layer of super glue adhesive. The coated disc was loaded between the two brushed surfaces of the rods using two V-blocks. The arrangement was left to be cured/ hardened for 5 minutes. Copper wires were introduced to the mentioned holes at the two rod sides. The wires of the specimen were clamped to the crossheads of the MTS M/C, as shown in Fig. 2. The maximum normal force applied on the coating sample, before being detached from the substrate, was measured. This force is considered as a measure for the adhesion strength/bond strength of the coating. In other words, it is the amount of force the coatings can withstand before being delaminated from the substrate. Three replicates from each sample were tested.

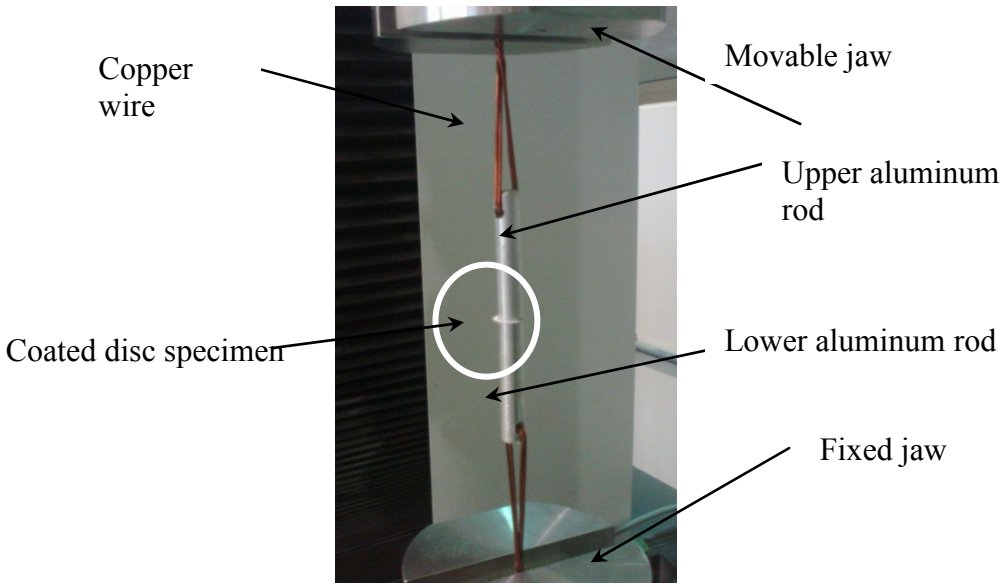

Fig. 2. Coating adhesion testing by MTS machine: coated samples were loaded as shown in the highlighted section of the apparatus.

Evaluation of Coatings' Topography. The surface topographies of the fabricated coatings were examined by scanning electron microscopy (SEM). The coated discs were mounted onto metal stubs and secured with adhesive carbon tape. The discs were sputter coated with gold to produce a film of 10 nm thick, and then werer being analyzed by Hitachi S-4000 SEM (Hitachi, Pleasonton, CA). The working voltage of 15 kV and working distance of 15 mm were set.

Investigation of the Thermal Properties of the Coatings

Differential Scanning Calorimetry (DSC) Analysis. A standard DSC (Perkin Elmer DSC-7) was used to determine the thermal characteristics of the optimally fabricated coatings. A sample of 10 to 20 mg, in an aluminium sample pan, was placed in the DSC under nitrogen atmosphere. The temperature and enthalpy calibration of the system was carried out using indium. Sample powders were carefully extracted from the coating specimens. Each tested specimen was held at room temperature for 5 min before heating to 160 °C at a controlled heating rate of 10 °C/min. The heat flow versus temperature (i.e., thermogram) was plotted and analyzed. Triplicates of each sample were tested under the same experimental conditions.

Thermogravimetric Analysis (TGA). The thermal decomposition/degradation of the developed nano-TiO_2-enriched coatings was studied by thermogravimetry in a PERKIN ELMER thermogravimetric instrument (TGA 7). Samples of a mass ranged between 10 and 20 mg were used for the tests. The samples were being heated from room temperature up to 450 °C in a dynamic air atmosphere with a flow rate of 60 ml/min and a heating rate of 10 ^{o}C/min in the thermal analyser. The degradation (i.e., % weight loss) of the samples was monitored and measured as a function of temperature and was analyzed with the TGA analysis program. The TGA thermograms and the first derivatives (DTG) were then plotted. Hence, the initial decomposition temperature (T_d) and the maximum decomposition rate temperature (T_{max}) were evaluated for each coating.

Cell-Surface Interactions. The developed nano-TiO_2-enriched coatings were disinfected and sterilized in preparation for tissue culture. The developed surfaces were rinsed twice with ethanol (70%), washed thrice with phosphate buffered saline (PBS) and then placed into individual wells of a 24-well tissue culture plate. The entire plate was then placed into a tissue culture hood and exposed to UV light (30 min) on each side of the samples to ensure sterility.

Human embryonic palatal mesenchymal cells (HEPM, ATCC CRL-1486) were seeded onto the coated surfaces in multiple 24-well tissue culture plates (50,000 cells/well). The cultures were maintained in Dulbecco's modified eagles medium (DMEM) supplemented with fetal bovine serum (FBS, 10%), L-glutamine (2 μmol/ml), penicillin G (100 U/ml), streptomycin sulfate (100 μg/ml) and amphotericin B (0.25 μg/ml). Replicate cultures were incubated at 37^{o}C for 6, 24 and 72 hours and then harvested for analysis.

Cellular interactions on the coated surfaces were examined by SEM. After 6, 24 and 72 hours of incubation, the replicate surfaces were collected, washed three times with PBS, fixed with glutaraldehyde (2.5%) in cacodylate buffer (100 mM) for 20 minutes. The fixed cells were then dehydrated in ascending grades of ethanol (i.e., 25, 50, 75, 95 and 100%) and immersed in hexamethyldisilazane. Then the surfaces were air dried inside the laboratory, mounted on metal stubs, sputtered with gold (20 nm thick) and examined with SEM. The working voltage (5-12 kV) and the working distance (5.3 mm) were set.

Cellular interactions on the developed nanoparticles-enriched coatings were also examined by inverted fluorescence microscopy. After 24 and 72 hours of incubation, the replicate surfaces were harvested and washed thrice with PBS. The cells that attached to the surfaces were fixed with paraformaldehyde (4%) for 10 minutes and permeabilized with Triton X-100 (0.1%) for 5 minutes. The actin filaments of the cytoskeleton were labeled with rhodamine phalloidin for 2 hours at room temperature. The surfaces were then mounted using Vectashield with DAPI and examined by an inverted fluorescence microscope with a magnification of 20X.

Results and Discussion

Coatings Development and Topography. Polymeric powder coatings were developed by applying well-processed polyester resin-based coating formulation consisting of micron-sized titanium dioxide (25%), nanoparticles of titanium dioxide (0.5%) and PTFE, fillers and additives through ultrafine powder coating technology [33-36]. Keeping other ingredients same, PTFE concentrations (in weight percent) of 3.0, 2.0, 1.0, and 0.0% were used to create PPC(3), PPC(2), PPC(1), and PPC(0) respectively. Variation of PTFE content in the coating formulations significantly changed their surface roughness/topographies [50]. SEM examination, as shown in Fig. 3, revealed that PPC(0) was very flat and even across its surface; PPC(1) was relatively smooth with only a few pits and projections; PPC(2) had more pits and projections than the previous two; and PPC(3) surface was composed of numerous pits, deep grooves, concavities and surface projections. Earlier study [35] claimed that PPC(0) and PPC(1) possessed nano-scale features/roughness, while PPC(2) and PPC(3) had submicron levels of surface roughness, which is also evident from the SEM images presented in Fig. 3. Atomic force microscope (AFM) images presented in [35] revealed similar features on the coating surfaces. Therefore, it is clear that with the ascending order of PTFE concentration (from 0 % to 3%), surface roughness and concavities gradually increased from PPC(0) to PPC(3) resulting in the marked changes in overall surface topographies of the developed coatings.

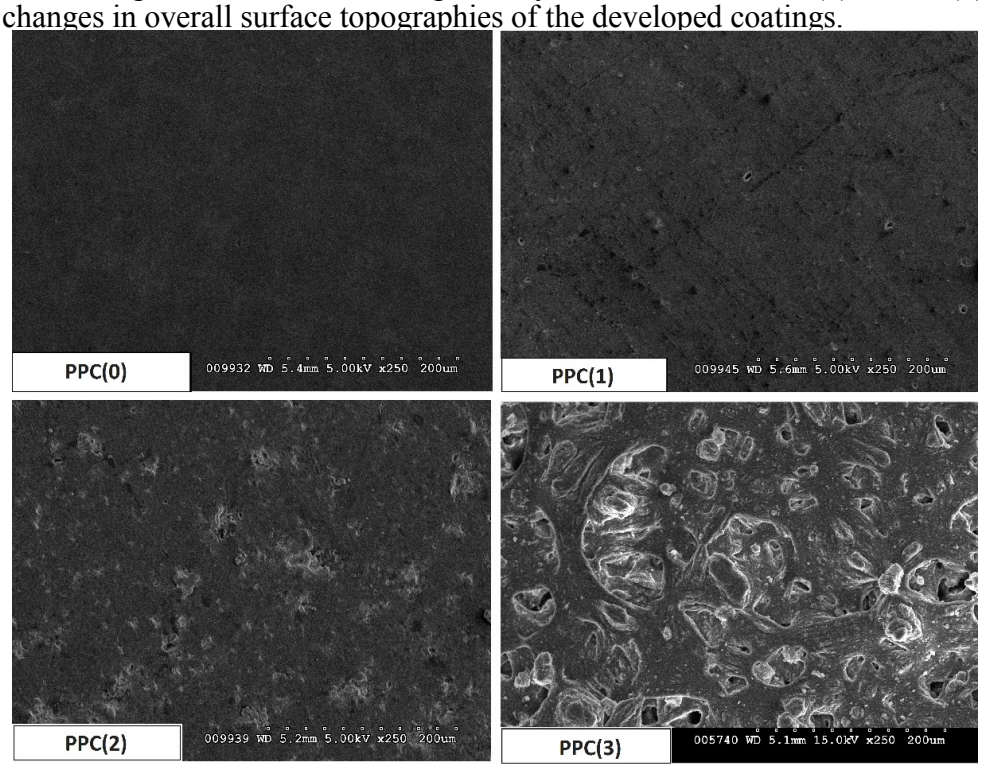

Fig. 3. SEM images of the developed coatings.

Coating Adhesion Strength. Given that PPCs of varying micro and nano-roughness were successfully created, it is essential that the polymeric coatings have to be strongly adhered to the

underlying substrates. In our previous studies [33-35], we reported a qualitative coating adhesion test according to ASTM D3359 standard. In this test, the coatings were cut vertically down to the substrate with a sharp blade, and multiple perpendicular cuts were made to create a grid of small squares. This lattice was then covered with an adhesive tape, and firmed with a pencil eraser. Then, within 2 minutes of application, the tape was pulled off with an angle of about 180° taking off the loose lattice with its way. The remaining lattice was then assessed to score from 0 – 5B for adhesion according to the ASTM standard D3359. And all the developed coatings were evaluated with 5B adhesion [35] which was the maximum in the range.

In this study, a novel coating sample loading system was designed and fabricated with the aim of completely eliminating all kind of lateral forces in the transverse direction while applying the load. The system was inserted in the MTS universal tensile testing machine. Then maximum load was recorded before the coating being detached from the metallic substrates. The experimental results, peak load and adhesion strength, were summarized in Table 1. The average value of at least three samples of each coating was reported. The standard deviation is also shown in the table. It is observed that, all the PPCs could withstand a normal force of over 775 N in magnitude. Hence, the adhesion strength was found to be over 5 MPa. Moreover, Fig. 4 shows two representative photographs of the same sample before (a) and after (b) being failed or detached under applied loads. It is evident from Fig. 4(b) that the amount of the coating delaminated from the substrate was not more than 20% of the total area of the disc and the middle of the coating was still intact. This further demonstrated the adhesion strength of the developed polymeric coatings. In addition, Table 1 confirms the decrease of adhesion strength with the increase of PTFE concentrations; this pattern is explained by that fact that hydrophobicity of the coatings increased with the increment of PTFE percentage [35]. Presence of higher concentration of PTFE (i.e., PPC(3)) in the polymer matrix increased the unmelted regions during the molten curing phase, hence increased the volume fraction of porosity resulting into poorer adhesion strength.

Table 1. Adhesion tests: peak loads and adhesion strength.

Samples	Peak Load [N]	Adhesion Strength [MPa]
PPC(0)	1553.84 ± 28.79	10.86 ± 0.2
PPC(1)	1443.22 ± 58.53	10.08 ± 0.4
PPC(2)	1215.18 ± 65.11	8.49 ± 0.45
PPC(3)	775.988 ± 2	5.4 ± 0.014

Fig. 4. Adhesion tests; (a) before and (b) after being delaminated from the metallic substrate.

The higher values of normal forces that the developed coatings tolerated could be attributed to the proper dispersion of nanoparticles [40,41], mechanical interlocking [41] and chemical crosslinking [40,42] of the polymers with other ingredients during the curing process of the coatings (i.e., coalescing the polymer particles to form a continuous film on the surface). Well dispersion of TiO_2 nanoparticles into the polymer matrix [33-36] minimized the porosity of the cured coatings resulting into enhanced mechanical interlocking and cohesiveness of the coatings. Moreover, lack of chemical crosslinking led to the increment of the viscosity of molten phase that caused reduction of the adhesion strength of the developed polymeric coatings.

Thermal Properties of the Coatings. DSC heating curves have been obtained for the developed composite coatings to determine their crystallization melting points and glass transition temperatures. It has been found that the glass transition temperatures for polyester and PTFE were 75 °C and 110 °C, respectively and the crystallization melting temperatures for polyester and PTFE

were 215 °C and 420 °C, respectively. According to the standards ISO 11357-2, ISO 11357-3 and ASTM D3418, the glass transition and melting temperatures for polyester are 60 °C and 223.8 and 225 °C, respectively; and the glass transition and melting temperatures are 115 °C [51] and 327 °C, respectively. Fig. 5 presents the representative DSC curves showing the glass transition temperature for PPC(0) and PPC(3). Moreover, PTFE has shown a very clear crystallization peak (not included in the figure). Then the heat of fusion of the crystallization peak of PTFE in the coatings has been measured to be approximately 168.3 J/g. since the composite coatings were primarily made of polyester, micro and nano-sized TiO_2 and PTFE, and cured at 200 °C, the observed deviation from the literature value is understandable. However, further studies are required in uncover the cure kinetics of the developed coatings

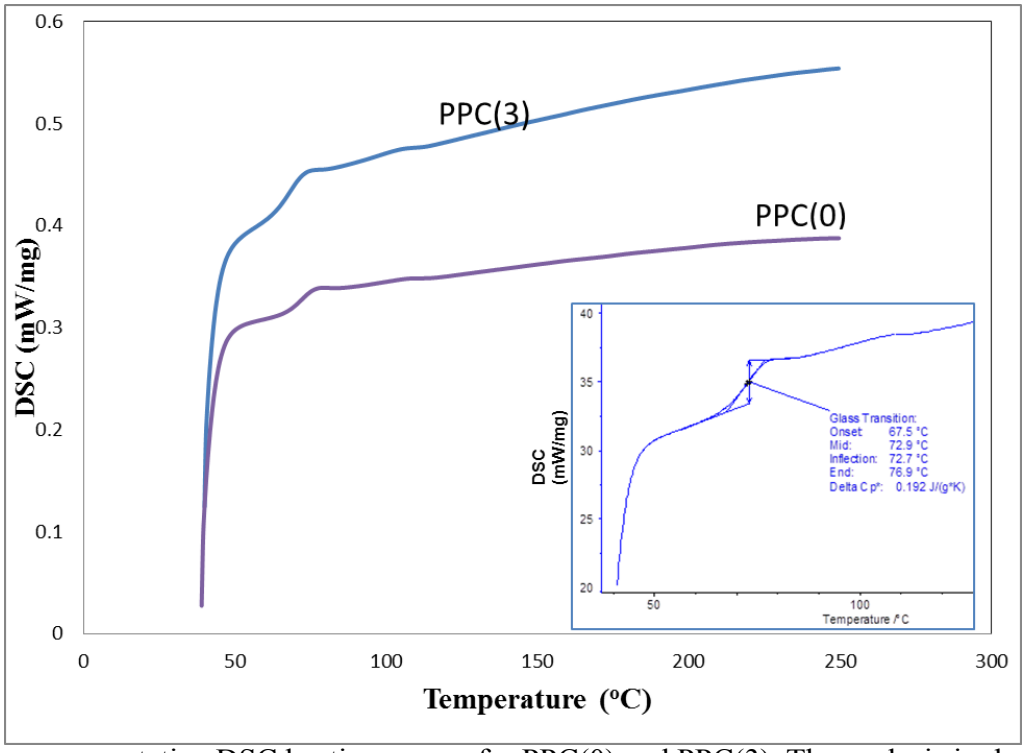

Fig. 5. The representative DSC heating curves for PPC(0) and PPC(3). The analysis is shown in the heating curve placed on the inset.

The thermogravimetric analysis (TGA) in a dynamic air atmosphere and derivative thermogravimetric analysis (DTG) were conducted on the coating samples to investigate their thermal stability. All measurements were made in duplicate with good level of repeatability. More details about the TGA technique may be found in Mourad et al. [43-49] for different polymeric materials. A typical representative TGA and DTG curves obtained from the device showing the different decomposition temperatures is presented in Fig. 6. The figure demonstrates how the onset/initial degradation temperature T_d (^oC) and the maximum rate of decomposition temperature T_{max} (°C) based on the device definition are obtained. The T_d (°C) was found at the intersection of the two tangents of 1st and 2nd TGA curves. The maximum rate of decomposition temperature T_{max} (°C) was found at the DTG curve peak.

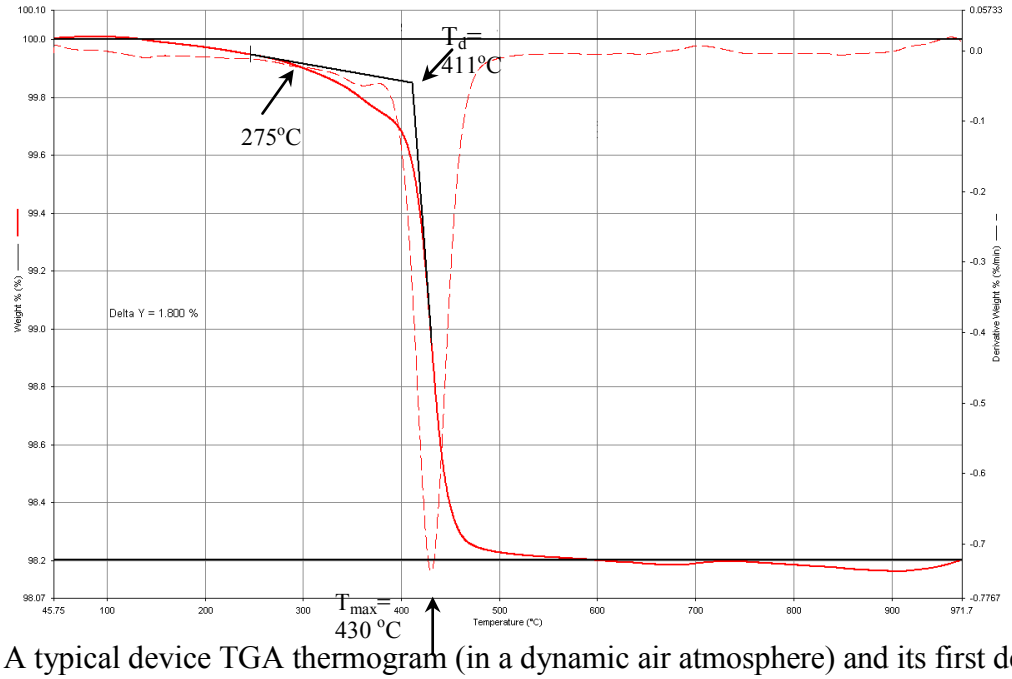

Fig. 6. A typical device TGA thermogram (in a dynamic air atmosphere) and its first derivative (DTG) for a representative sample showing the onset of decomposition T_d (=411 °C) and maximum decomposition rate temperature T_{max} (=430 °C).

It is worth noting here that the weight loss hasn't reached 100% as the coating was on a metallic substrate which will not be decompose under the heating cycle involved; moreover, the developed coatings contain metallic nanoparticles. That is, the weight of the coating represents part of the sample which is approximately from 2% to 4 % of the total weight of the sample. Therefore the weight losses at the end of the test were not 100%.

It is observed that all samples started to decompose at T_d not less than 400 °C (for PPC (0)) and the maximum T_d value is 423 °C and the maximum rate decomposition temperature T_{max} ranges from 430.3 °C to 442.4 °C. The coating of PPC (0) has the lowest values. It is known that the thermal stability in a specific application depends on the operating temperature and exposure time. The usual temperature for the coating process is around 200 °C and the coating will be exposed to human body temperature (~40 °C) after implantation. The relatively high decomposition temperatures observed for the developed composite coatings indicated their thermal stability in a wide range of operating temperatures.

Cell Attachment and Growth. In the previous studies [33-36], we reported that nano-TiO_2-enriched polymeric coatings supported human embryonic palatal mesenchymal (HEPM) cells' attachment, spreading and growth, although the biological performances of the developed coatings markedly differed depending on their surface topographies and micro-/nano-features. HEPM cells attached and spread on some of the PPCs as early as 6 hours of seeding [35], while enhanced cell growth and proliferation were observed on all PPCs after longer incubation period (i.e., 1 and 3 days) [33-36]. Cell growth and proliferation (from 6 hours to 3 days) are summarized in Fig. 7 and 8 [35]. It is evident that more cells were attached on PPC(0) compared to the other PPCs. It is interesting that although the adhesion and thermal properties of all the PPCs were similar, the remarkable differences in cell growth were observed due to the presence of nano features (PPC(0)) and sub-micro scale roughness (PPC(1), PPC(2), PPC(3)). Similar studies reported that polymeric substrates (i.e., UHMWPE, PTFE, polyurethane etc.) enriched with a nano-sized titanium supported enhanced osteoblast cell responses [4-6].

The polymeric powder coatings that were of similar surface roughness exhibited similar degree of cellular responses [33-34], while variation in surface roughness/features were largely linked to the marked differences in cellular responses [35]. More specifically, the coatings consisting of nano-scale features, resulting into nano-roughness (<100 nm), supported excellent HEPM cell growth, proliferation and osteogenic differentiation, whereas the coatings having sub-micron-range surface topographies supported comparatively less cellular responses than the former coatings [35]. Similar results were reported by Chun et al. [32] when poly(ether) urethane (PU) and PLGA surfaces were etched to create submicron and nano-scale surface features, the nano-rough surfaces supported higher urothelial cell densities. Similarly, polyurethane with a thin nano-rough film (<100 nm) of

titanium or zirconium was found to support enhanced human umbilical vein endothelial cell attachment and proliferation [52]. Moreover, Ranjan et al. [9] fabricated micron-patterned nano-rough poly(dimethylsiloxane) (PDMS) films that enhanced endothelial cell adhesion and elongation. Indeed, the nano-rough polymeric coatings [35] provided significantly larger surface area that in turn facilitated greater protein adsorption resulting in enhanced cell attachment and growth [53].

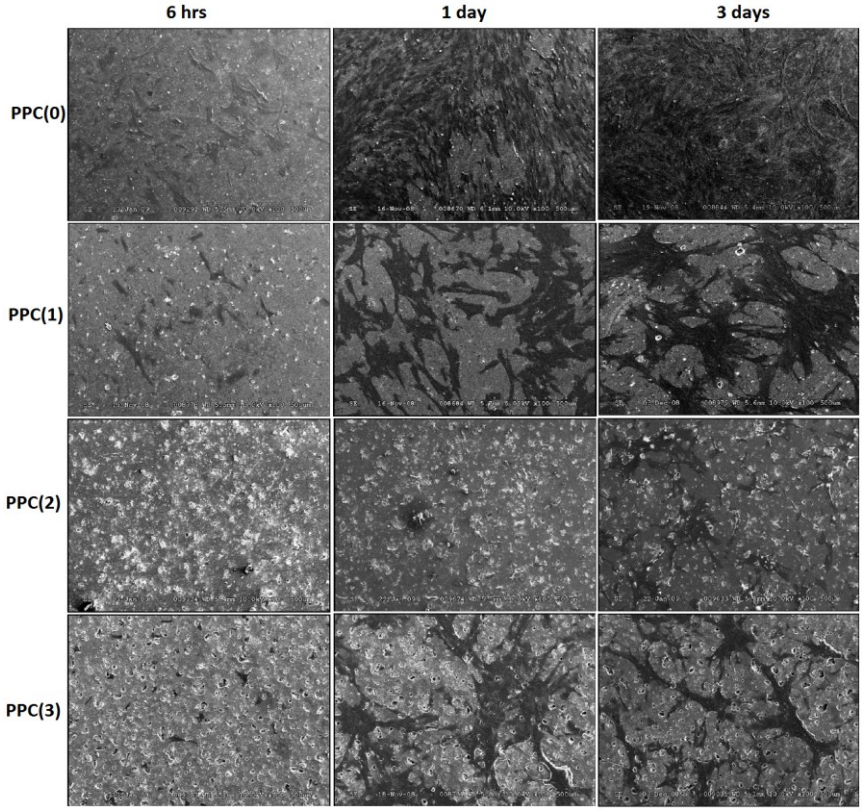

Fig. 7. SEM images of HEPM cells attached to PPCs starting from 6 hours to 3days (reproduced from [35]).

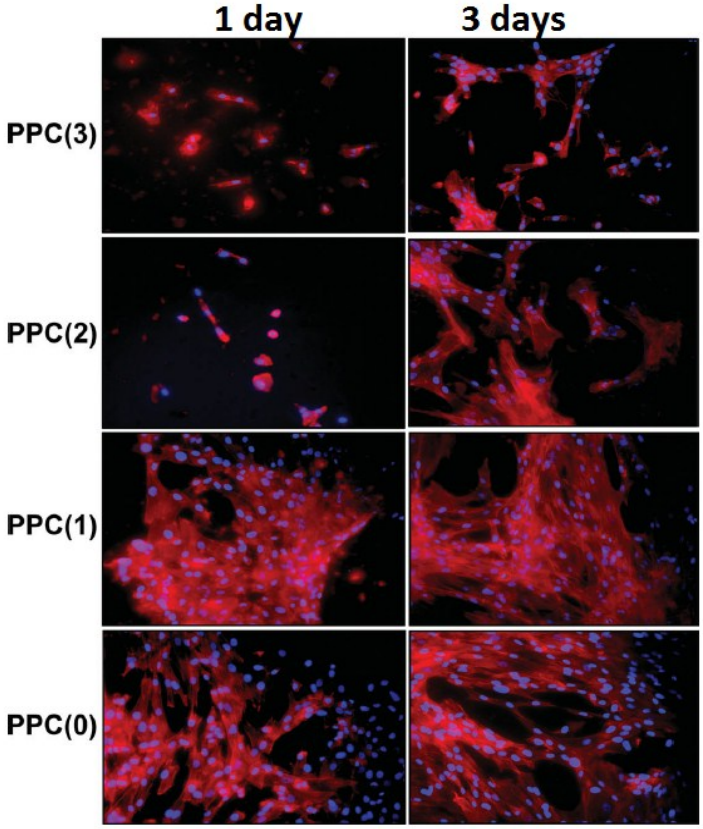

Fig. 8. Immunofluorescence images of HEPM cells attached to PPCs after 1 and 3 days (reproduced from [35])

Conclusion

Dependencies of cellular responses on the implants‘ nano-topographycal features and surface roughness were reconfirmed in our studies that are in complete agreement with the reports that have shown that enhanced cell attachment, growth and osteogenesis. Furthermore, this study has introduced a novel appratus to quantitatively measure coating adhesion to its substrate. We have found ecxellent coating adhesion strength for all tested PPCs (over 5 Mpa) which supports our previous qualitative finding on coating’s adhesion [33-35]. Moreover, coatings thermal properties were also investigated through TGA and DSC techniques that turned out with similar results for all PPCs. The melting, glass transition and thermal decomposition temperatures were found to be closer for all samples suggesting their usage in human body environment. However, coatings‘ cure kinetics needs to studied in detals in order to better understand their thermal perperties.

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Nanomaterials in Electrochemical Biosensor

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Abstract. Nanomaterial based electrochemical method gain tremendous interest for the detection of biomolecules due to high sensitivity, selectivity, and low fabrication cost. High surface to volume ratio, excellent electrocatalytic properties of the nanomaterials plays important role for the sensitive and selective detection of biomolecules. For electrochemical biosensors, proper control of chemical, electrochemical and physical properties, as well as their functionalization and surface immobilization significantly influences the overall performance. This chapter gives an overview of the importance of the development of nanomaterials based electrochemical biosensors; particularly direct electrooxidation- or electroreduction-based biosensors, catalysis-based biosensors, and label-based affinity biosensors. In addition, fabrication methods including modification of electrode surface with nanomaterials, tailoring their physico-chemical properties, and functionalization with chemicals or biomolecules are also highlighted.

Introduction

Sensing of biomolecules is very important for our health and safety. For example, catecholamine is an important class of biomolecules which works as a neurotransmitter. Specific areas our body should contain certain level of this molecule. Abnormalities in the amounts of this neurotransmitter lead to several diseases such as Parkinson's disease [1]. Glucose is another important biomolecule which acts as a principle source of energy in the human body. However, the presence of glucose in blood outside the normal range of 80-120 mg/dL (4.4-6.6 mM) is a sign of diabetes [2]. As a result, various methods have been developed for sensing biomolecules, such as colorimetric, spectrometry, quartz crystal microbalance, high performance liquid chromatography, and electrochemical techniques. Among all these methods, electrochemical method became more popular due to its ease of operation, fast response time, low cost, good sensitivity and selectivity. Electrochemical biosensors can be classified into three major subsections on the basis of its working principles, such as; (i) direct electrooxidation- or electroreduction- based biosensor (ii) catalysis- based biosensor, and (iii) affinity biosensor. In case of direct electrooxidation or electroreduction technique, comparatively higher electroactive molecules can be measured on certain working electrode surfaces. On the other hand, most of the catalysis-based biosensor depends on the enzymes. The immobilized enzymes on the electrochemical transducer's surface generate electroactive species in the presence of the target analyte and the enzymatic product is electrochemically oxidized or reduced to determine the target analyte. For affinity-based electrochemical biosensor, a specific biological recognition event is used to trigger an electrochemical response; the analytes are generally antigen,

nucleic acid (DNA and RNA), biomarker, etc. The affinity-based electrochemical biosensor can be further classified as label free and label-based biosensor. Label-free method has several advantages over the label-based biosensor, such as, less time consuming, low cost, free from biofouling, *i.e.* nonspecific binding. However, its high detection limit is the principle barrier for early diagnosis of diseases. On the other hand, the label-based electrochemical biosensor became more popular because of its low detection limit. Again, getting a low detection limit partially depends on the type and amount of labels, and also on low nonspecific binding of label molecules with the sensing surface. In the label-based electrochemical method, label materials can work as a direct electrocatalyst or catalyst. As catalyst, the label lead the conversion of an electroinactive substrate (S) molecule to an electroactive product (P) which can be involved in an electrochemical reaction in sensing potential window to generate a signal with an electrochemical product (EP). The basic principle of a label-based biosensor is described in Fig. 1. The sensitivity of this type of label-based biosensor partially depends on the amount of generated electroactive 'P' from the electroinactive 'S'.

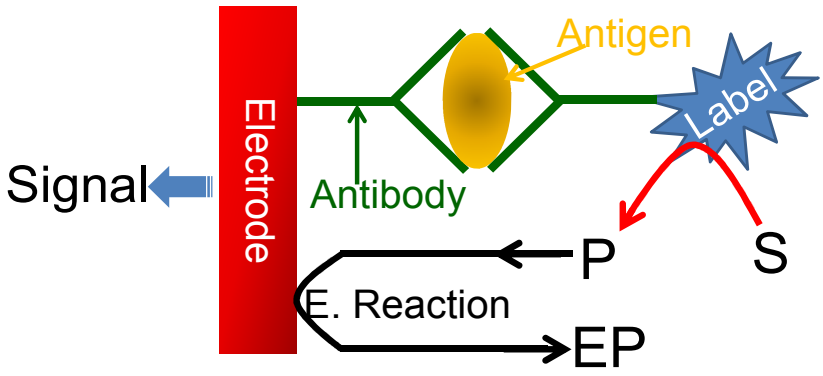

Fig. 1. Schematic of label-based electrochemical immunosensor.

Generally, enzymes are used as labels in electrochemical biosensors. However, some researchers have also used nanomaterials as efficient labels [3-15] for direct electrocatalyst/catalyst or to anchor other electrocatalyst/catalysts including other small nanomaterials and enzymes or to act as a seed for increasing the size of labels. Nonspecific binding, which is the main obstacle to get a low detection limit in label-based biosensor, can be minimized by incorporation anti-biofouling layer on the sening surfaces [16, 17]. Sensitivity and selectivity of electrochemical biosensors partially depends on the properties of the working electrodes, such as electrocatalytic property, conductivity, stability, etc. Most of the conventional working electrodes in biosensors show poor electrocatalytic properties toward large numbers of electroactive molecules. These poor electrocatalytic properties should be improved in order to obtain high sensitivity and selectivity in biosensors for the effective diagnoses of diseases in early stages. A lot of researches have been done to improve the electrocatalytic properties of the conventional electrodes and particularly, nanomaterial based improvedment, because of its unique properties, such as good electrocatalytic properties, high conductivity, stability, biocompatibility, easy of surface functionalization, etc. Several reviews of nanomaterials-based biosensors are available [11-15, 18]. The aim of this chapter is to review the important developments, including recent inventions in nanomaterial-based electrochemical biosensors covering modification of electrode surfaces with nanomaterials, electrocatalytic properties, functionalization of nanomaterials for direct electrooxidation or electroreduction-based biosensors, catalysis-based biosensors, and label-based affinity biosensors fabrication.

Direct Electrooxidation or Electroreduction-based Electrochemical Biosensors

Only selective electrode materials are suitable for the electroxidation or electroreduction of target molecules, for this reason, different scientists developed suitable electrode materials for specific target biomolecules [19, 20, 22-40, 42-46]. They also paid attention on fabrication methods of electrode material for better reproducibility, stability, robustness and low cost.

Hydrogen peroxide (H_2O_2) is an important biomolecule as it is involved in various processes, such as signaling cascades, protein hydrolysis, and maintenance of the redox homeostasis in a cell [21]. High levels of H_2O_2 are closely associated with cancer and progressive neurodegenerative diseases, such as Parkinson's disease [19]. In addition, it is widely used in food, pharmaceuticals, paper, and chemical industries [19]. As a result, there have many opportunities to enter the H_2O_2 into living organism. In past decades, enzyme-based electrochemical H_2O_2 biosensors were popular due to their good sensitivity and selectivity. However, the application of enzyme-based H_2O_2 biosensors is limited due to some serious disadvantages, such as complicated enzyme immobilization procedures, the ease of enzyme denaturing, instability of enzyme-modified electrodes, etc. To overcome those limitations, many scientists developed nanomaterial-based nonenzymatic H_2O_2 biosensors. For example, the Jiang group developed a nonenzymatic H_2O_2 biosensor using the good electrocatalytic properties of CuS/RGO nanocomposites [19]. They simply heated the mixture of $CuCl_2$ and Na_2S (aq.) in the presence of poly(vinyl pyrrolidone) (PVP)-protected graphene oxide at 180 °C to produce a CuS/RGO nanocatalyst. Next, they modified GCE by simply casting the alcoholic solution of the developed nanocatalyst on a GC electrode, and subsequent drying. The cyclic voltammograms showed that CuS/RGO-modified GCE is able to reduce the H_2O_2 at a more positive potential with a higher current than that of bare GCE or PVP-modified GCE or RGO-modified GCE [Fig. 2] *i.e.,* CuS/RGO-modified GCE possesses excellent electrocatalytic properties for H_2O_2 reduction. The CuS/RGO-modified electrode shows good detection limit (0.27 μM), stability, and fast response for the determination of H_2O_2. Finally, they used this composite electrode to detect H_2O_2 in serum and urine samples, and also in the living cells.

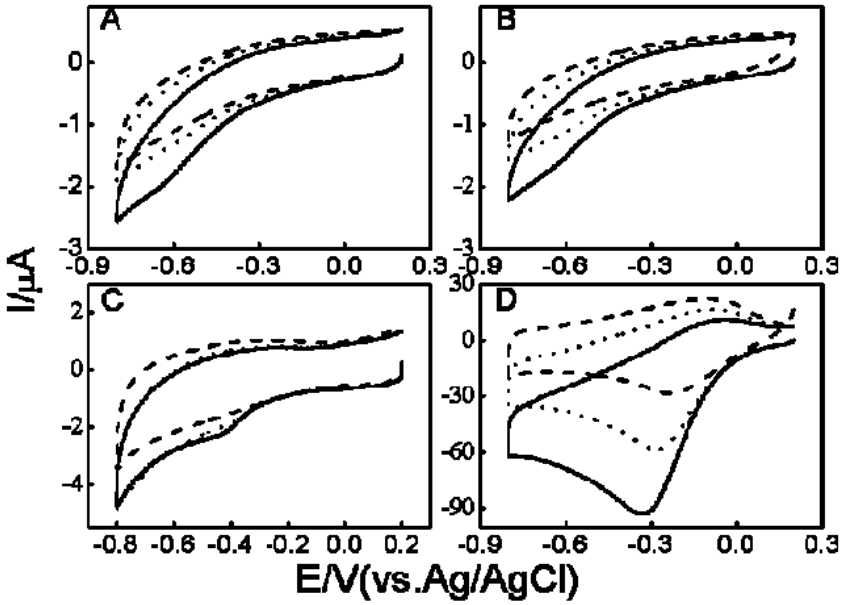

Fig. 2. Cyclic voltammograms obtained in 0.2 M phosphate buffer solution (pH 7.4) containing 0 mM (dash line), 2 mM (dot line), and 5 mM (solid line) H_2O_2 at GC (A), PVP/GC (B), RGO/GC (C) and CuS/RGO/GC electrodes (D) at scan rate of 50 mV/s. Reprinted with permission from reference [19] with the permission from American Chemical Society.

Aziz *et al.* [20] developed a Pd nanoparticle-modified graphite pencil electrode (PdNP/GPE) for the nonenzymatic amperometric determination of H_2O_2. They prepared the modified electrode by immersing the bare GPE in to an aqueous solution of pre-synthesized PdNP, and heated at 75 °C. The fabricated PdNP/GPE showed better electrocatalytic properties toward the electrochemical reaction of H_2O_2 in both cyclic voltammetry and amperometry experiments (Fig. 3). The detection limits were 45 nM and 0.58 mM, respectively, for the PdNP/GPE and bare GPE in amperometric determination at + 0.8 V. However, the higher detection limits were obtained on both electrodes with applied potential of -0.25 V than that of +0.8 V. In addition, the PdNP-GPE showed improved analytical selectivity, sensitivity, and stability. Similarly, platinum hollow nanospheres/polypyrrole nanowire/GCE [22] and CuNP/GCE [23] were reported as excellent electrode materials for the sensitive electrochemical detection of H_2O_2.

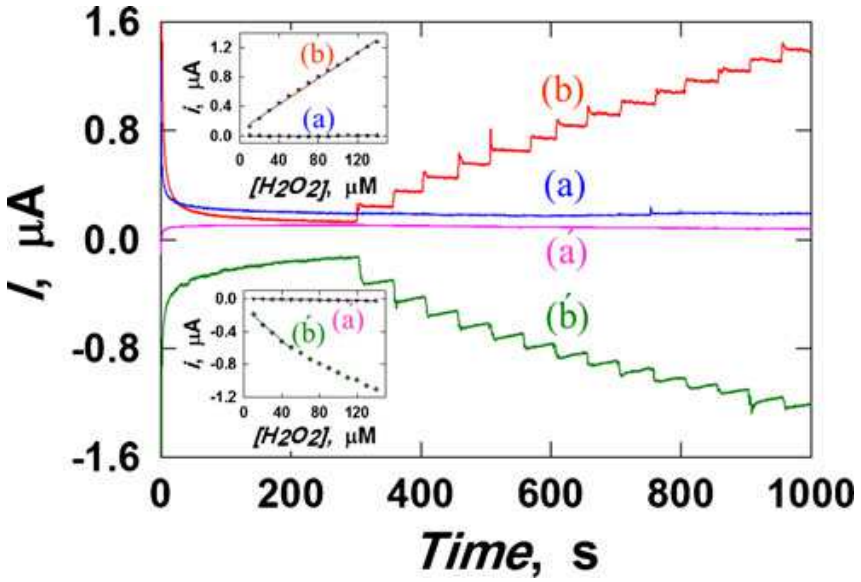

Fig. 3. Amperograms of bare GPE (a, a') or PdNP/GPE (b, b') electrodes in 10 mL PBS (0.1 M, pH 7) at +0.80 V (a, b), during the successive addition of 10 μM of H_2O_2 and at -0.25 V (a', b'), during the successive addition of 50 μM H_2O_2. Amperograms (a, b) and (a', b') were recoded before and after purging with argon for 20 min, respectively. The insets show the corresponding calibration plots. Reprinted from reference [20] with kind permission from Springer Science.

Though glucose sensor is a well established technology, still there are few challenges with the electrode materials [24-26]. Recently, Zhong *et al.* [27] have demonstrated a nonenzymatic glucose sensor based on the excellent electrocatalytic properties of Pd@Cysteine-fullerene(Pd@Cys-C_{60}) nanoparticles. Pd@Cys-C_{60} nanoparticles were prepared in two steps using a spontoneous reduction process without any additional reductant (Fig. 4)**.** In this process, C_{60} was functionalized with Cys and the Pd^{2+} ions were captured via co-ordination bond on the functionalized C_{60} followed by *in situ* spontaneous reductions to form Pd@Cys-C_{60} nanoparticles. The purified and re-dispersed solution of the Pd@Cys-C_{60} nanoparticles were dropped on the GCE and subsequently dried for electrochemical evaluation. The Pd@Cys-C_{60}-modfied GCE showed excellent electrocatlytic properties toward the glucose oxidation. The fabricated glucose sensor allowed a low detection limit (1 μM) with a linear range from 2.5 μM to 1.0 mM in the amperometric detection method. Interestingly, no observable interference was observed in the current response of glucose from the potential interferents such as

ascorbic acid, uric acid, *p*-acetamidophenol, and fructose. These anti-interfering properties might be attributed from the low working potential (-0.05 V) in the experiment. This sensor also showed good reproducibility and stability.

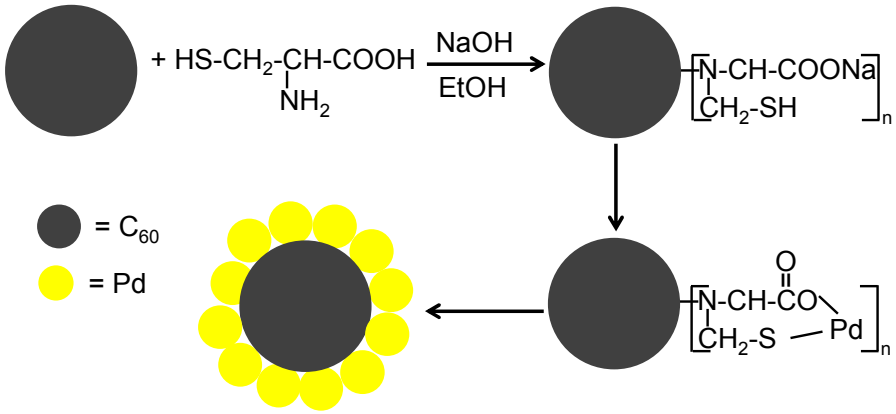

Fig. 4. Synthetic scheme of Pd@Cys-C_{60} nanoparticles.

To further improve the detection limit in the glucose sensor, Zhang *et al.* [28] developed a reduced graphene oxide-nickel nanoparticles composite on GCE (NiNP/RGO/GCE). Initially, they dropped the solution of GO on GCE and after drying the GO was reduced electrochemically. Then they deposited NiNP electrochemically from Ni precursor (Ni_2SO_4). They also observed that the morphology of the developed nanocomposite depends on the deposition time of Ni (Fig. 5). All these fabricated NiNP/RGO/GC electrodes (different Ni deposition times) were tested for glucose detection. The NiNP/RGO/GCE with 50 s deposition time, showed high stability and sensitivity, was chosen as a working electrode for the glucose sensor. Detection limit in the amperometric method at NiNP/RGO/GCE was 0.1 µM with a wide linear range from 2 µM to 2.1 mM (R=0.996). Similarly, the fabrications of non-enzymatic glucose sensors were described on the basis of excellent electrocatalytic properties of Au nanowire [29], AuNP [30], CoOOH nanosheet [31], and Pt/Ni-Co nanowire [32].

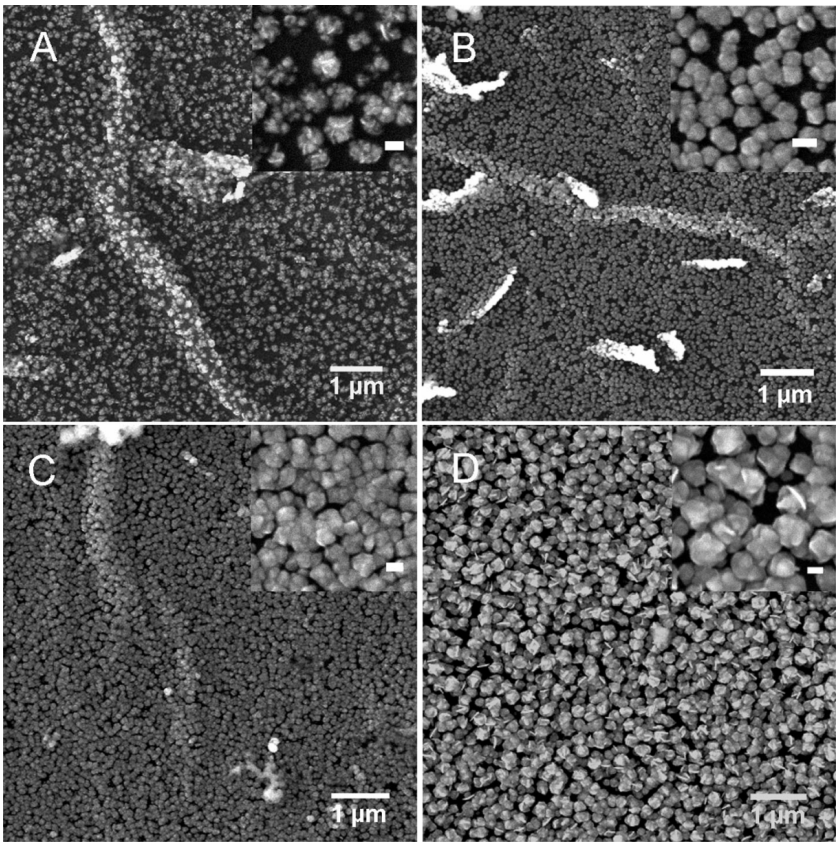

Fig. 5. SEM images of the NiNPs/RGO/GCE with different deposition times: 10 s (A), 50 s (B), 100 s (C), and 200 s (D). Applied potential: 1.0 V. Scale bar of the inset images: 100 nm. Reprinted with the permission from Elsevier [28].

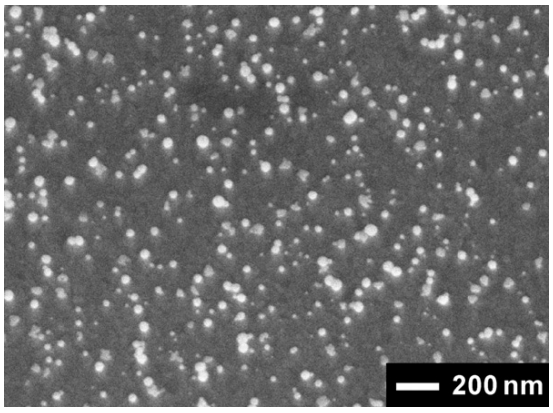

Fig. 6. A FE-SEM image of the surface of a densely coated AuNPs on ITO electrode. Reprinted with the permission from Elsevier [33].

AuNP has also been used for the electrochemical detection of *norepinephrine* (NE) which is an important neurotransmitter [33]. For detection of NE, the AuNP was simply deposited on an indium tin oxide (ITO) electrode by immersing the bare electrode in an aqueous solution of $HAuCl_4$ and ascorbic acid, and subsequently heated at 75 °C [33]. The AuNPs were homogeneously distributed on ITO, and the particles sizes were in the range 20-40 nm (Fig. 6). The AuNP/ITO showed excellent electrocatalytic properties toward electrochemical detection of NE with a very low detection limit 87 nM. The developed sensor was also highly selective for the detection of NE in the presence of ascorbic acid, dopamine, and uric acid, which are the common interferents in complex matrices such as biological fluids (*e.g.,* urine and plasma). Due to high sensitivity and selectivity, the developed AuNP/ITO was applied to detect NE in human blood and urine samples. They observed good stability and reproducibility of this electrode as well. Another important neurotransmitter is *epinephrine*, which was detected on AuNP-attached multi-walled carbon nanotube-layered ITO (AuNP/MWCNT/ITO) electrode [34]. The AuNP/MWCNT/ITO shows much better electrocatalytic properties toward epinephrine and *p*-acetamidophenol than that of AuNP/ITO and MWCNT/ITO alone. The low detection limit, high reproducibility, selectivity and simplicity are the attractive features of this work. The AuNP/MWCNT/ITO was also used for monitoring the effect of *p*-acetamidophenol on the release of epinephrine in human urine. Similarly, another important neurotransmeter, *dopamine* was selectively and sensitively detected on a palladium nanoclusters /polyfuran film/Pt [35], Pt@Au/MWNTs/GC [36], graphene-AuNP nanocomposite film/GC [37], graphene nanosheet/SnO_2 NP hybrid nanocomposites/GC [38] electrodes.

Aziz *et al.* [39] prepared and used the AuNP/MWCNT/ITO electrode for the electrochemical detection of *tryptophan*, which is an important and essential amino acid for humans and herbivores. The electrode was able to oxidize tryptophan at a low potential with a high oxidation current, which is essential to get high sensitivity, selectivity, and a low detection limit. The electrode showed a 25 nM detection limit for tryptophan. Finally, the authors applied this electrode for the determination of tryptophan in human urine and plasma samples using the standard addition method. An electrochemical tryptophan sensor was also developed by Mao *et al.* [40] using a Ag@C core-shell nanocomposite on GCE (Ag@C/GCE). They made the Ag@C nanocomposite by a simple hydrothermal method [41], which was casted on a GCE electrode. They showed the effect of C shell for protecting the Ag core and the contribution of enhanced substrate accessibility and tryptophan substrate interactions, at the same time the nano Ag core can display good electrocatalytic activity toward tryptophan. The linear sweep voltammetry technique was applied for the concentration dependence study, and obtained a 40 nM detection limit for tryptophan. The sensor showed high selectivity for the determination of tryptophan in the presence of interferents cysteine, lysine, valine, leucine, serine, threonine, histidine, isoleucine, ascorbic acid, uric acid, and dopamine. Finally, Ag@C-GCE was applied for the determination of tryptophan in an amino acid injection and in rat blood serum using the standard addition method.

The reliable monitoring of ascorbic acid (vitamin C), is imporatnt for clinical diagnostics, sport medicine, biotechnology, cosmetics, and food analysis. Zhang *et al.* [42] developed a highly sensitive and selective ascorbic acid sensor based on the accelerated electron transfer reaction of ascorbic acid at a carbon-supported PdNiNPs-modified GC electrode. The low (-0.05 V) applied potential in the amperomeric method was sensitive enough to detect ascorbic acid with high selectivity. On the other hand, a FePt alloy NPs-modified electrode was used for sensitive and selective determination of ascorbic acid in the presence of common interference species, including dopamine, citric acid, uric acid, glucose, and NaCl [43]. The selective determination was achieved due to low over potential for electrooxidation of ascorbic acid at a FePt alloy NPs-modified electrode. Similarly, the excellent electrochemical properties of AuNPs@polyaniline core-shell nanocomposites/GCE were used for the fabrication of sensitive and selective ascorbic acid sensors [44].

Detection of cholesterol plays an important role in the biomedical field and in improving the quality of life as its abonormal levels lead to the symptoms of several diseases, such as hypertension, coronary heart disease, arteriosclerosis, brain thrombosis, lipid metabolism dysfunction, and

myocardial infarction abnormalites. However, there have been many reports of the enzymatic electrochemical detection of cholesterol. Although enzymatic base sensors showed good sensitivity, selectivity, and reproducibility, their practical application are limited due to their high cost, instability, and complicated enzyme immobilization procedure. To overcome these limitations, Li *et al.* [45] developed a nonenzymatic method for the electrochemical detection on the basis of excellent electrocatalytic properties of porous tubular AgNPs. They prepared porous tubular AgNPs by simple electrodeposition of silver NPs on a CdS-modfied porous anodic alumina (PAA) template, and subsequent removal of CdS. The porous tubular AgNPs were cast on the GC electrode for their electrochemical charecterization and application in a nonenzymatic electrochemical cholesterol sensor. The porous tubular AgNPs-modfid GCE showed obvious electrocatalytic properties for electrooxidation of cholesterol whereas bare GCE showed no electrocatlytic properties for cholesterol electroxidation (Fig. 7). The fabricated sensor gave 0.18 mM detection limit in the amperometric detection method. The sesnor was selective for cholesterol determination in the presence of glucose, uric acid, glycerol, and β-estradiol.

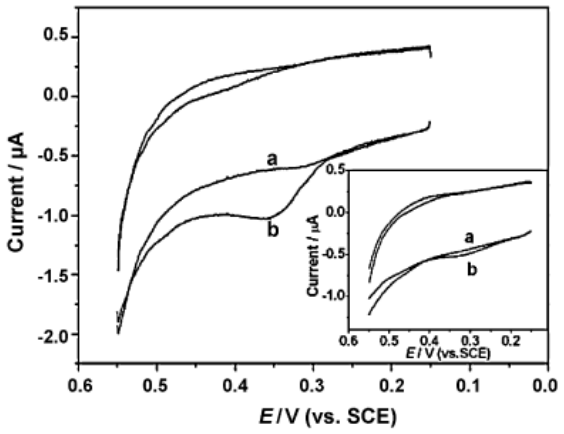

Fig. 7. Cyclic voltammograms of bare GCE (inset) and porous AgNP-modified GCE in the absence (a) and presence (b) of 7.4 mM cholesterol in 0.1M NaOH at 100mV/s. Reprinted with the permission from Elsevier [45].

In another work, Deng *et al.* [46] used a nanocomposite (Nafion/MWCNT-chitosan-AuNP) on GCE for the electrochemical determination of nitric oxide (NO), which is a very important biomolecule. NO plays an important role in mediating vasoconstriction, signal transmission, apoptosis, immunity, and gastrointestinal motility. A Nafion/MWCNT-chitosan-AuNP film on GCE was prepared by a simple drop-drying method. They examined the effect of the film thickness, applied potential, and volume of Nafion on the electrochemical signal. It showed a good detection limit of 7.6 nM with high selectivity. Finally, the developed sensor was successfully applied to monitor NO release from living tissues, including mouse kidney, heart, spleen, and liver (a slice).

Catalysis-based Electrochemical Biosensors

This type of biosensor basically depends on electrocatalytic activity of the enzymes. Although nonenzymatic biosensors have many advantages over enzyme-based biosensors, which have been discussed in the earlier section, some nonenzymatic biosensors need harsh conditions, such as high pH (aqueous solution of NaOH) which some time hinder many real sample analyses. Moreover, many target analytes cannot be oxidized or reduced at low potential for their selective and sensitive determination on the non-enzymatic electrode surfaces, whereas the immobilized enzyme on the suitable electrode surface can generate electroactive molecules which can be electrochemically

oxidized or reduced for signal generation at low potential in neutral pH or near to neutral pH. Conventional electrodes like the GC electrode are not suitable enough for the electrooxidation or electroreduction of enzymatic product at low potential. As a result, the immobilization of an electrocatalyst or electron mediator on a conventional electrode is required prior or during the immobilization of the enzyme. For example, dihydronicotinamide adenine dinucleotide (NADH), which is an enzymatic product of 300 NAD^+ dependent dehydrogenase enzymes [47], can be oxidized on a Fe_3O_4 nanoparticles/MWCNT-modified GC electrode in neutral pH media at 0 V (vs. Ag/AgCl), which is 650 mV lower than that of the bare GC electrode [48]. Teymourian *et al.* [48] combined this excellent electrochemical property with the excellent enzymatic properties of lactate dehydrogenase (LDH) for developing the sensitive and selective lactate biosensor. The modification procedure of sensing electrode surface, and the mechanism of bioelectrocatalytic sensing of lactate using the modified electrode are described schematically in Fig. 8. Similarly, Aydogdu *et al.* [49] developed the ethanol biosensor using a NiO nanoparticles-modified carbon paste electrode and ethanol dehydrogenase enzyme. NiO nanoparticles-modified carbon paste electrode electrooxidized NADH at significantly lower potential with higher current in neutral pH compared to that of bare GCE, *i.e.,* the NiO nanoparticles-modified carbon paste electrode showed excellent electrocatalytic properties which lead to the detection of ethanol in neutral pH.

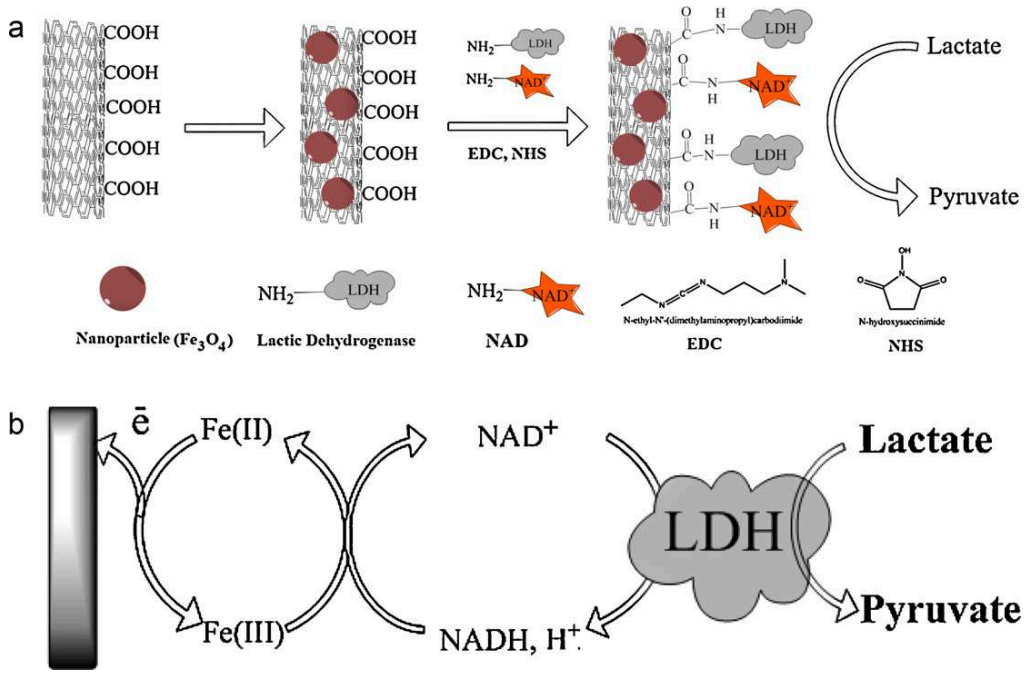

Fig. 8. Schematic representation for (a) the fabrication of NAD^+ /LDH /Fe_3O_4/MWCNT- modified GC electrode and (b) the mechanism of bioelectrocatalytic sensing of lactate using this modified electrode. Reprinted with the permission from Elsevier [48].

On the other hand, H_2O_2 is the enzymatic product of numerous enzymes, such as L-lactate oxidase, choline oxidase, etc. As a result, detection of H_2O_2 is very important for sensing biomolecules such as lactate, choline, etc. The nonenzymatic detection of H_2O_2 on the basis of the excellent electrocatalytic properties of nanomaterial-modified conventional electrodes were described earlier as the electrocatalytic properties of a conventional electrode are not sufficient for the electrochemical oxidation or reduction of H_2O_2 at lower over potential. As a result, coupling of

the enzymes with a nanomaterials-modfied electrode, which possesses good electrocatalytic properties toward the electrochemical reaction of H_2O_2, is logical for the sensing of biomolecules such as lactate, choline, and glucose. Accordingly, Zhang *et al.* [50] prepared Fe_3O_4 magnetic NPs and a choline oxidase-modified gold electrode for a choline biosensor. The enzyme choline oxidase produces H_2O_2 in the presence of analyte choline chloride. The reduction peak of the produced H_2O_2 appeared at 0.37 V in a buffer solution (pH 8.0) (*vs.* Ag/AgCl) on a Fe_3O_4 magnetic NP-modified Au electrode. The author showed sensitive (detection limit 0.1 nM) and selective detection of choline chloride on the basis of the excellent electrocatlytic properties of Fe_3O_4 magnetic NPs. Using the same principle, a glucose sensor had been fabricated using glucose oxidase and PdNP-decorated MWCNT-modified GCE [51]. Here, glucose oxidase produces H_2O_2 in the presence of glucose. The generated H_2O_2 was sensitively and selectively detected on a PdNP-decorated MWCNT-modified GC electrode for the sensitive and selective detection of glucose.

Label-based Electrochemical Affinity Biosensor

Nanomaterials play three important roles in a label-based electrochemical affinity biosensor. First, they can be used as electrocatalysts or electron mediators for improving the elctrocatalytic properties of a substrate electrode as discussed earlier. Second, nanomaterials allow an efficient docking site for immobilizing biomolecules in several ways, such as nonspecific adsorption and covalent immobilization. Finally, they can be used as labels for the generation of signals. By considering the first two roles, Aziz *et al.* [16] developed a sensitive immunosensor using an MWCNT-modified ITO (MWCNT/ITO) electrode. Simply, they prepared the MWCNT/ITO by immersing the ITO electrode in an aqueous solution of carboxylated MWCNT solution and successively washed and dried it. The MWCNT/ITO electrode showed low background current. However, it showed significantly improved electrocatalytic properties toward electrooxidation *p*-aminophenol (AP) compared to a bare ITO electrode. It is notable that the AP is the enzymatic product of a popular enzyme substrate, *p*-aminophenyl phosphate (APP), in biosensor. By the way, the surface coverage of the MWCNT layer was only ca. 0.03, *i.e.,* this modified electrode has a lot of free space which can lead to nonspecific binding of label molecules, *i.e.,* biofouling. The biofouling is a big obstacle to getting a low detection limit in a label-based biosensor. As a result, the vacant spaces of the modified electrodes were covered with poly(ethylene glycol) (PEG)-silane copolymer, which has non-biofouling properties [52, 53]. Beside this, the side walls of MWCNT were also used to immobilize the protein *avidin*, which was used to capture biotinylated antimouse IgG. Subsequently, the target mouse IgG was immobilized on the biotinylated antimouse IgG. Finally, label alkaline phosphatase enzyme (ALP)-conjugated antimouse IgG was bound to the target mouse IgG. The bound ALP catalyzed the conversion of APP to AP which was electrooxidized on the MWCNTs to generate a signal. The complete immunosensor fabrication procedure with detection principle is described in Fig. 9. The sensor showed high selectivity and a low detection limit (10 pg/mL of mouse IgG). Due to its high sensitivity and selectivity, this scheme was adopted for the fabrication of a microchip-based immunosensor [54]. It is well known that microchip-based biosensors are widely used in point-of-care testing as it requires a small volume of samples. The developed immunosensing microchip consists of an upper plate made of poly(dimethylsiloxane) (PDMS) which was prepared according to a previous report [55], and a lower plate made of a modified ITO micropatterned glass. The selective modification of a working electrode band with carboxylated MWCNT, a reference electrode band with IrO_xNP, and the rest of the empty surface of the ITO micropatterned glass with PEG-silane copolymer, and its sensing procedure was described in detail at reference [54]. In this immunosensing microchip, a 3.5 µl sample solution was sufficient to obtain a low detection limit (10 pg/ml mouse IgG).

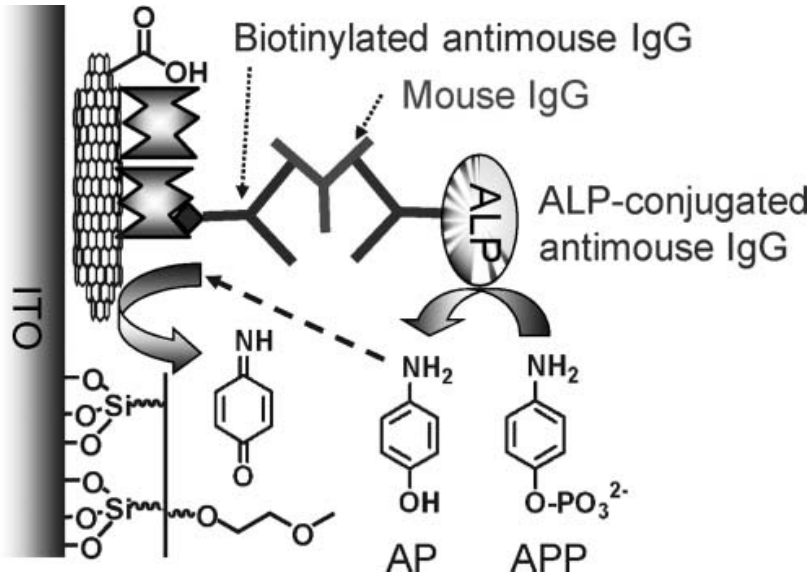

Fig. 9. Schematic illustration of an electrochemical immunosensor for detecting mouse IgG.

Reproduced from reference [16] with permission from The Royal Society of Chemistry.

For its excellent electrochemical biosensing properties, a PEG-silane copolymer/MWCNT/ITO electrode was also used to fabricate DNA sensors [17]. To fabricate DNA sensors, streptavidin instead of avidin was immobilized as an avidin-coated surface shows high nonspecific binding of DNA. The high isoelectric point of avidin (pI=10-10.5) is the principle cause of high nonspecific binding of negatively charged DNA under neutral pH. In addition, a streptavidin-coated surface allows low nonspecific binding of DNA [56]. However, the immobilization of streptavidin on solid surfaces through nonspecific binding is not suitable for biosensor fabrication. Besides, the covalently immobilized streptavidin on the PEG-silane copolymer/MWCNT/ITO sensing layer via the carboxylic group of MWCNT and the amine group of streptavidin is not enough to remove the nonspecific binding, as MWCNT has many vacant sides where the detection probe or label molecules can be bonded nonspecifically. As a result, the MWCNT surface was covered with an amphiphilic polymer which has two major sites, the hydrophobic dodecyl group and the hydrophilic PEG group [17, 57]. The hydrophobic dodecyl group and hydrophilic PEG group acted as anchoring group with CNT and protein-resisting group, respectively. Next, streptavidin was immobilized covalently through the carboxylic group of the amphiphilic polymer/PEG-silane copolymer/MWCNT/ITO surfaces and amine group of streptavidin to capture biotinylated capture probe DNA. Afterward, the target DNA (encoding residue 1038 of exon 11 of the BRCA1 gene) was hybridized with the capture probe. Then, the biotinylated detection probe DNA was hybridized with target DNA to capture ALP-conjugated streptavidin as label molecules. The sensing mechanism is similar to the immunosensor using the PEG-silane copolymer/MWCNT/ITO electrode. The explanation of the fabricated DNA sensor is illustrated in Fig. 10.

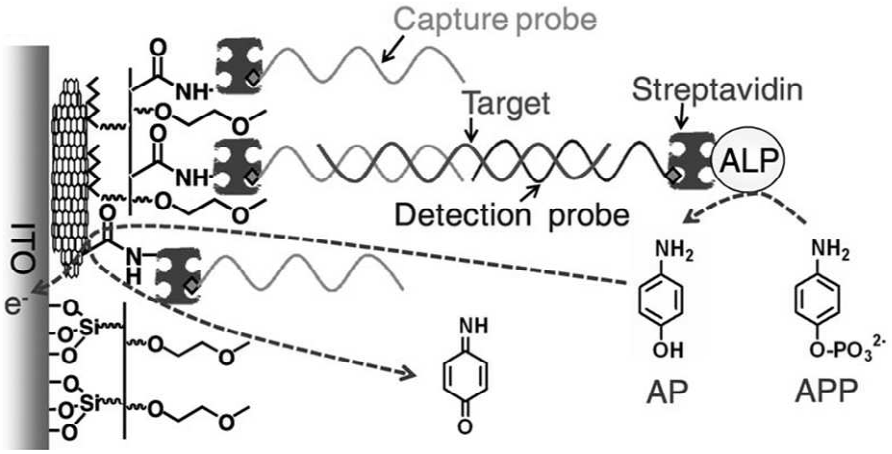

Fig. 10. Schematic sensing scheme of the electrochemical DNA sensor. Reprinted with the permission from John Wiley and Sons [17].

Similar to the immunosensor using the PEG-silane copolymer/MWCNT/ITO electrode, an ALP label-based immunsensor was fabricated without using any polymer, where AuNP was used as an electrocatalyst instead of MWCNT [58]. AuNP was prepared by capturing $AuCl_4^-$ ion electrostatically on a positively charged amine functionalized ITO electrode and successive reduction with ascorbic acid. The surface coverage of the AuNP on the ITO electrode was very low. However, low surface coverage of Au nanoparticles on an ITO electrode showed low background current, and high electrocaltyic properties toward AP oxidation. In addition, the AuNP gave efficient sites for the immobilization of avidin. The other part of the fabrication of the immunosesnor was similar to the immunosensor fabricated using the PEG-silane copolymer/MWCNT/ITO electrode. The immusensor fabrication with sensing mechanism is illustrated schematically in Fig. 11. Interestingly, the immunosensensor showed almost no nonspecific binding and high selectivity with a low detection limit (10 pg/ml) for mouse IgG.

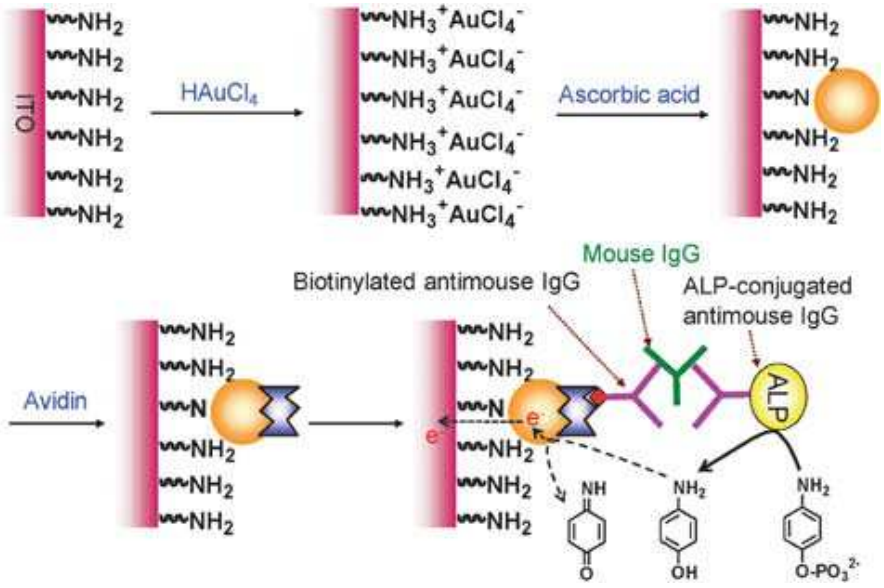

Fig. 11. Schematic representation of the formation of AuNPs on an amine terminated ITO electrode, and the preparation of an electrochemical immunosensor for detecting mouse IgG. Reproduced from reference [58] with permission from The Royal Society of Chemistry.

Loading amount of enzyme on a secondary antibody or biotin is limited, as a result, this kind of label can give a limited number of catalytic sites for the conversion of an electroinactive enzyme substrate to an electroactive product. However, the sensitivity depends partially on the amount of generated electroactive species in this type of biosensor. Besides, nanomaterials have high surface to volume ratios, which might be applied to load a large number of enzyme-conjugated secondary antibodies or other proteins, and this bioconjugated nanomaterial can be used as a label in a biosensor for generating large amounts of electroactive molecules. For example, Yang *et al.* [3] used horseradish peroxidase-secondary antibodies-conjugated nanographene oxide as a multi-labeled and biocompatible probe to increase the electrochemical response in an immunosensor. In this scheme, they reduced the enzymatically oxidized thionine in the presence of H_2O_2 on an AuNP-attached nitrogen-doped graphene sheet-modified GC electrode for signal generation. They clearly demonstrated that the horseradish peroxidase-secondary antibodies-conjugated nanographene oxide label generated a much higher signal compared to that of using only horseradish peroxidase-secondary antibodies as a label for the same concentration of target analyte matrix metalloproteinase-2, which is one of the key biomarkers in blood [3]. The developed immunosensor showed excellent analytical performance with quite a low detection limit (0.1 pg/ml matrix metalloproteinase-2). Similarly, the Wang group developed a magnetic bead-based ultrasensitive immunosensor and DNA sensor using the ALP-conjugated CNT as label and α-naphthyl phosphate as enzyme substrate [4]. The enzymatic product, α-naphthol was accumulated and oxidized on a CNT-modified GC electrode to achieve an extremely low detection limit.

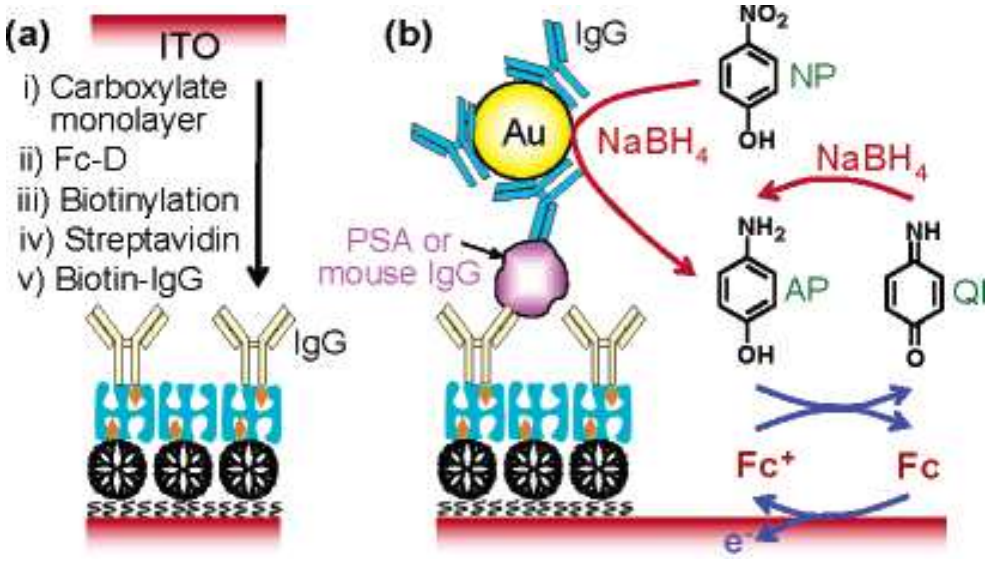

Fig. 12. Schematic representation of the formation of AuNPs on an amine terminated ITO electrode, and the preparation of an electrochemical immunosensor for detecting mouse IgG. Reprinted with the permission from American Chemical Society [5].

Interestingly, only nanoparticles were used as a label instead of an enzyme by Yang group [5]. They fabricated immunosensors based on catalytic reduction of *p*-nitrophenol to AP using gold-nanocatalyst labels [5] and $NaBH_4$ as reductant. The produced AP was oxidized to *p*-quinone imine (QI) on a ferrocenyl-tethered dendrimer-modified ITO electrode to generate the electrochemical signal, and the electrochemical signal was further amplified by chemical reduction of QI by $NaBH_4$. The fabrication procedure with sensing mechanism of the ultrasensitive immunosensor is presented in Fig 12. The obtained detection limit was 1 fg/mL for both mouse IgG and prostate specific antigen. With the same principle, the Chen group [6] developed an alpha-fetoprotein immunosensor using an AuNP-modified CNT as label instead of using only AuNP as label, and a thionine-modified GC electrode as sensing electrode instead of a ferrocenyl-tethered dendrimer-modified ITO electrode. The result of their work was the generation of more AP from *p*-nitrophenol using the large catalytic site of a large number of AuNP on one CNT compared to that of using a single AuNP, *i.e.,* further amplification of a signal to achieve a lower detection limit.

On the other hand, the single NP or multi NP-decorated nanomaterial can be used as label in an affinity- type biosensor, whereas a label molecule can act as an electrocatalyst or electron mediator for electrochemical signal generation on poor electrocatalytic electrode materials. For example, Polsky *et al.* [7] used PtNP label as an electrocatalyst and an Au electrode as sensing electrode materials, and H_2O_2 as signal generating molecules for developing affinity-based DNA and thrombine sensor. As an Au electrode has poor electrocatalytic properties toward the electrochemical reduction of H_2O_2, the PtNP label acted as an electrocatalyst for the reduction of H_2O_2 to generate the electrochemical signal in the biosensor. Fig. 13 describes the analytical procedure of analyte sensing using PtNP as label. With this same principle, the Yang group [8] developed a highly sensitive electrochemical DNA sensor using chemically activated AuNP as label, ITO as electrode materials, and hydrazine as signal generating molecules. Due to the poor electrocatalytic properties of ITO, and high electrocatalytic properties of chemically activated AuNP toward electrooxidation of hydrazine, the chemically activated AuNP was used as efficient label in the DNA sensor [8]. Similarly, Li *et al.* [9] fabricated a sensitive biosensor for the detection of tumor necrosis factor-α using Prussian blue-functionalized ceria nanoparticles as label and GC as electrode material. The Prussian blue NP-functionalized ceria nanoparticles label generate signals from the electrochemical reduction of H_2O_2. In addition, PdNP-decorated carbon nanotube as label and gold nanoparticles

decorated graphene nanosheet-modified screen printed carbon as electrode material have also been used to fabricate a highly sensitive immunosensor [10]. The label acted as an electrocatalyst toward the reduction of dissolved oxygen to generate the signal in the immunosensor.

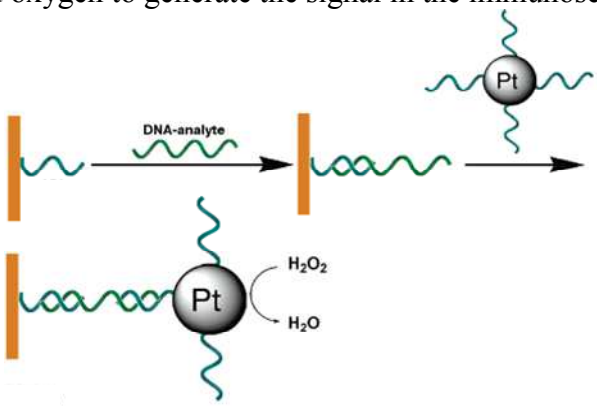

Fig. 13. Scheme depicting the analytical procedure for using the PtNPs in the analysis of DNA.

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Conclusion

Nanomaterial undoubtedly plays an important role in the sensitive and selective electrochemical detection of biomolecules in different biosensors. Here, we have briefly presented the various methods for the modification of electrode surfaces with the nanomaterials to obtain high signal amplification and reduce over potential in electrochemical reactions to obtain low detection limits with high selectivity. Moreover, we discussed the immobilization of biomolecules, including protein, nucleic acids, and enzymes on the large surfaces of nanomaterials, which is an important issue in biosensor fabrication. In addition, the use of NP as label was discussed in regard to further signal amplification in electrochemical biosensors to obtain ultrasensitive detection limits with high selectivity. According to our observation, there have been many improvements in the fabrication of sensitive and selective electrochemical biosensors. However, there are still more challenges yet to commercialize the developed nanomaterial-based electrochemical biosensor. These challenges include (i) the detection of biomolecules in a real sample which contains numerous numbers of interferences, (ii) a miniaturization system which requires a small volume of sample, and (iii) a large scale-multiplex detection of biomolecules. Moreover, new nanomaterials as electrode materials or label can further increase the sensitivity and selectivity in electrochemical biosensors. Therefore, there are still many things to develop in nanomaterial-based electrochemical biosensors for improving the quality of life as well as commercial purpose.

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