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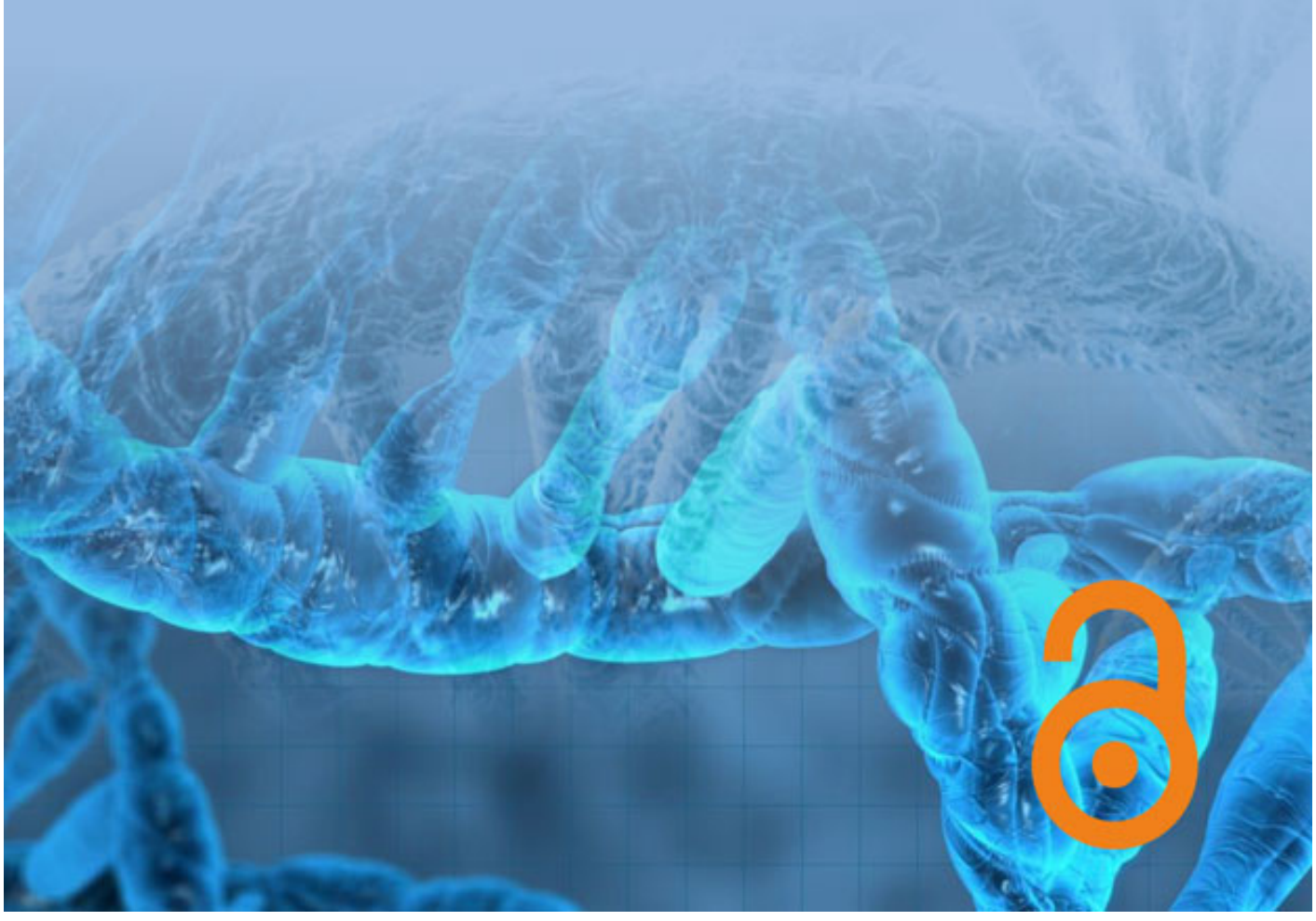

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Advances in Biochemistry & Applications in Medicine

Chapter 1

Advanced glycation end products (AGEs)-mediated diabetic vascular complications

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1. Introduction

Diabetes mellitus is a group of metabolic disorders leading to defects in insulin secretion and action of insulin or both. Diabetes is caused by a combination of hereditary and environmental factors. In the human body, blood glucose levels are controlled by a complex interaction of multiple chemicals and hormones, including insulin and glucagon. Insulin is a peptide hormone produced in the beta cells of the pancreas that allows blood glucose to enter various cells of the body where it is oxidized to yield energy needed by the muscles and tissues to function [1]. Glucagon is also a peptide hormone, produced by the alpha cells of the pancreas, which causes a rise in the concentration of glucose in the blood. The effect of glucagon is opposite to that of insulin, which lowers the glucose concentration.

There are three main types of diabetes, namely: Type 1 diabetes mellitus (T1DM), Type 2 diabetes mellitus (T2DM) and gestational diabetes (GDM). T1DM is a chronic autoimmune disorder that occurs in genetically susceptible individuals by environmental factors. In this condition, the body's own immune system attacks the pancreatic β-cells, and destroy or dam-

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age these cells to an extent where they are not able to meet the body's insulin requirements. T2DM is a metabolic disorder that is characterized by hyperglycemia (high blood sugar) in the context of insulin resistance and relative lack of insulin. GDM is a condition in which women without previously diagnosed diabetes exhibit high blood glucose levels during pregnancy (especially during their third trimester).

The global prevalence of diabetes, especially T2DM, is increasing at an alarming rate. According to the recent update by the International Diabetes Federation (IDF) more than 382 million adults aged 20-79 years had diabetes in 2013 [2].The prevalence is increasing in every country, and major economic, social and health care impacts will be seen in developing countries, as these countries are home to as much as 80% of people with diabetes. If these trends continue, by 2035, some 592 million people, or one adult in 10 will have diabetes, according to data of IDF (Fig 1) [IDF, sixth edition. 2013]. This equates to approximately three new cases in every 10 seconds or almost 10 million per year. Diabetes caused 5.1 million deaths in 2013 and every six seconds a person dies from diabetes. Diabetes is rampant in Indian subcontinent. India is the 2nd topmost country having the highest number of people with diabetes. In India alone, it is estimated that the total number of people with diabetes in 2013 was around 65.1 million, rising to 109 million by 2035.

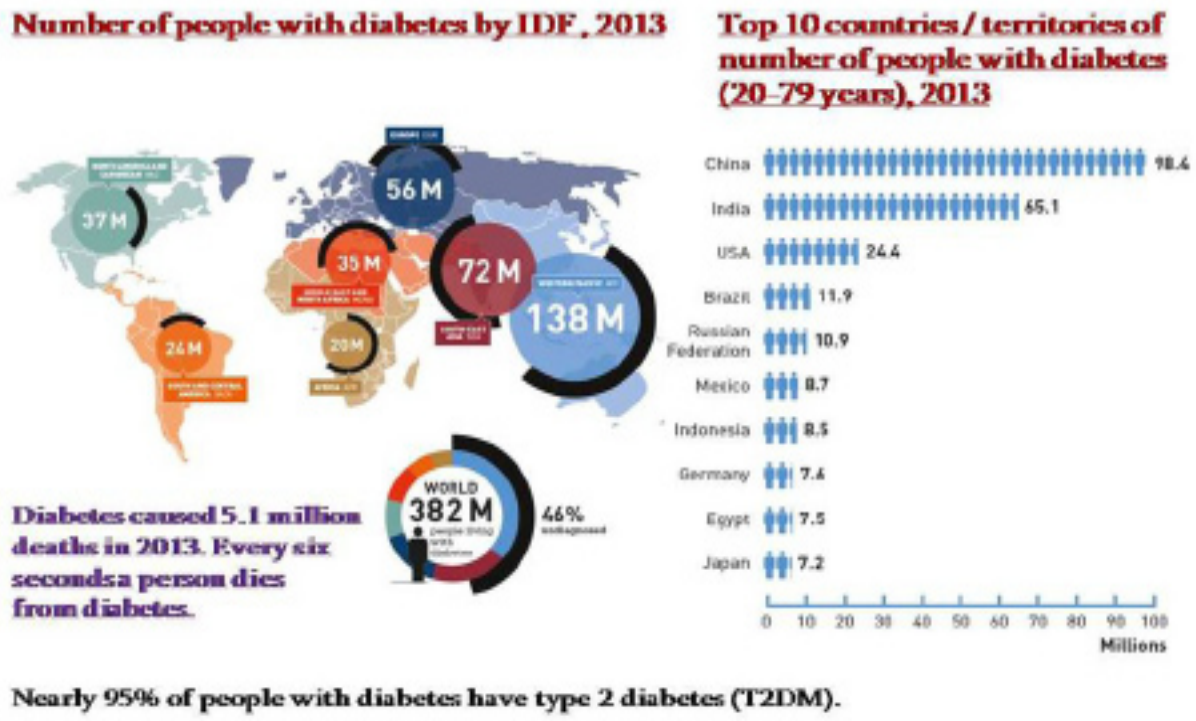


Figure 1: Global prevalence of diabetes mellitus (Adapted from Guariguata et al [2])

1.1. Vascular complications of diabetes

Microvascular and macrovascular complications manifesting as chronic vascular complications of diabetes, which are the major causes of morbidity and mortality (Fig 2). Diabetes especially type 2 diabetes (due to increased prevalence) has become the principal cause

of blindness and end stage renal disease. About 30-45% of all diabetic subjects suffer from microvascular complications. Patients with diabetes are at two to four times increased risk of coronary heart disease, cardiovascular disease and related deaths than those in the general population. Patients with diabetes are at four times higher risk of developing peripheral vascular disease (PVD) [3].

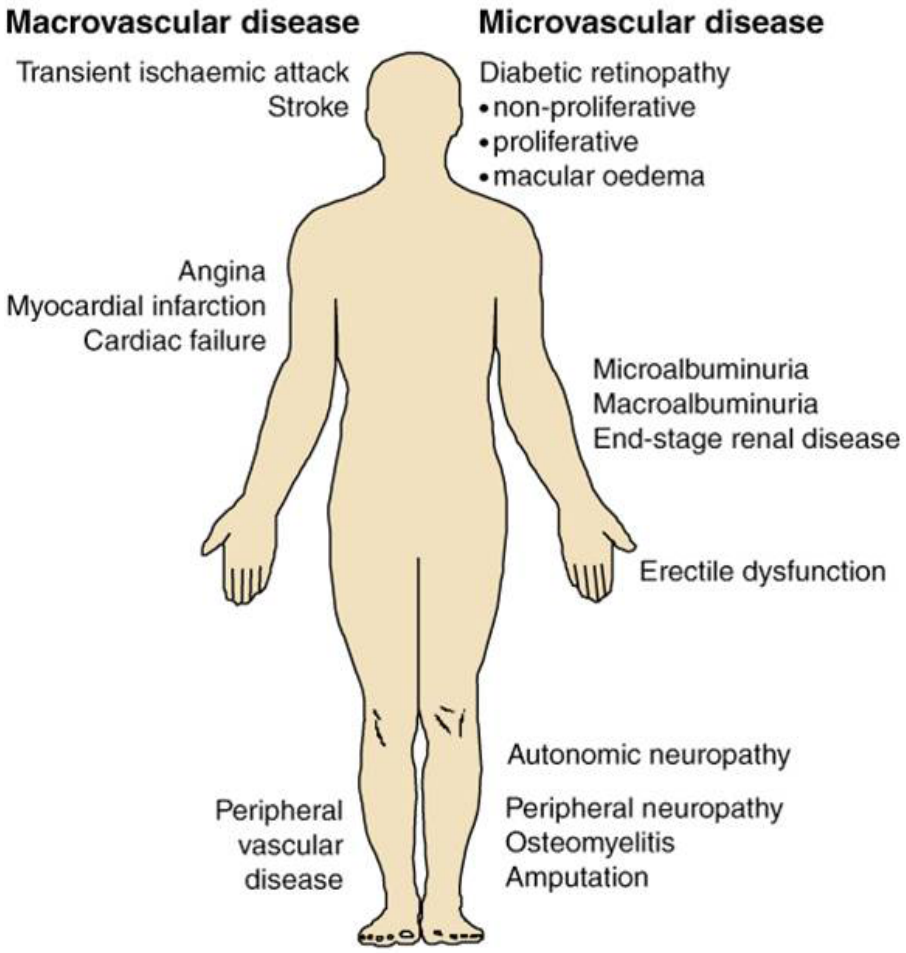


Figure 2: The major diabetic complications (Adapted from Bate et al [3])

The Diabetes Control and Complications Trial (DCCT) and United Kingdom Prospective Diabetes Study Trial (UKPDS) have clearly demonstrated the vital importance of intensive glycemic control in preventing the progression of diabetic complications. Hyperglycemia induces a variety of metabolic changes, which includes activation of polyol pathway, activation of the diacylglycerol protein kinase, and increased oxidative stress (Fig 3). Hyperglycemia inflicts cumulative long-term structural and functional changes in important macromolecules through advanced glycation end products (AGEs). Endothelial dysfunction in diabetes is most often described in the context of oxidative stress. This chapter summarizes the role of AGE-RAGE-oxidative stress system in diabetic vascular complications and its therapeutic interventions.

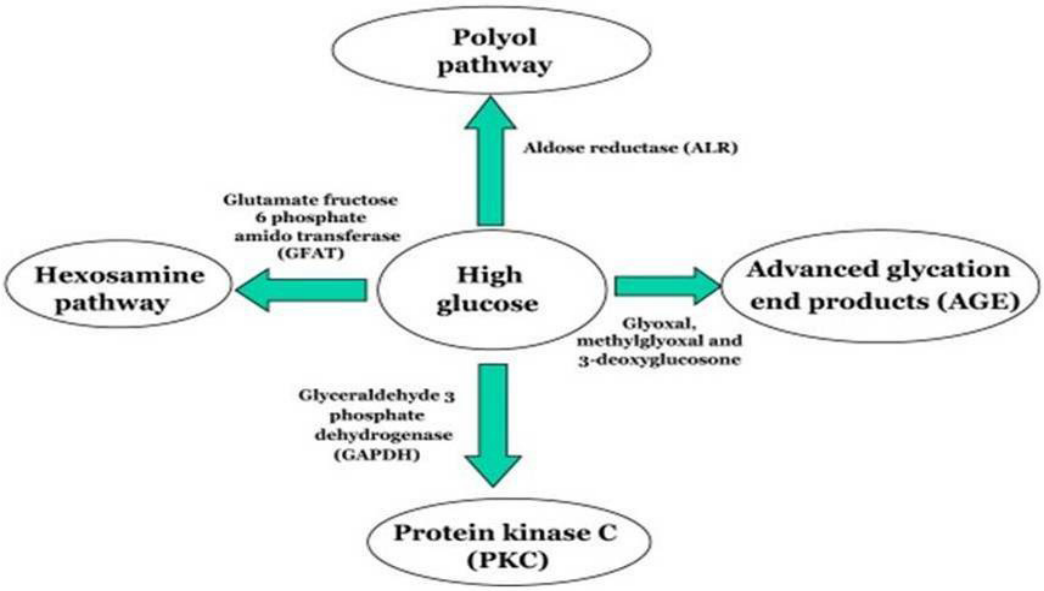


Figure 3: Hyperglycemia and its mechanism (Adapted from Brownlee M [8])

1.2. Hyperglycemia and oxidative stress

Oxidative stress (OS) is an imbalance between the production of free radicals and the body's antioxidant defense system. Free radicals are atoms or molecules that contain one or more unpaired electrons. Oxygen alone or either associated with hydrogen ion or nitrogen, can be converted into very highly reactive molecules, including OH radicals, superoxide, NO, H_2O_2 etc., which rapidly interact with proteins, lipids and carbohydrates [4]. These highly reactive molecules attach to the normal cellular components, changing them into abnormal ones. Thus a critical cell membrane protein can become very rigid instead of being flexible, which impairs cell functions and may lead to cell death giving rise to various complications (Fig 4) [5].

Free radicals are formed disproportionately in diabetes by glucose oxidation, non-enzymatic glycation of proteins, and subsequent oxidative degradation of glycated proteins [6]. Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes, increased lipid peroxidation, and development of insulin resistance. Therefore OS is considered a common endpoint of chronic disease like diabetes [7] and is often characterized by an increase in superoxide and hypochlorous acid. OS can modulate a wide variety of biological processes by coupling signals at the cell surface with changes in gene expression, suggesting the multiple signaling pathways are involved [8]. Indeed, reactive oxygen species (ROS) may be defined as true second messenger molecules that regulate various signal transduction cascades upstream of nuclear transcription factors, including modulation for Ca^{2+} signaling protein kinase and protein phosphate pathways [8].

At physiologic levels, oxidants are important signaling molecules that are involved in processes such as cell growth and regulation of transcription factors like nuclear factor-kappa B (NF-kB). Normally the cell regulates the amount of oxidants very closely. The amount of oxidants in the cell is determined by the balance of production of oxidants versus the destruction of oxidants (called reduction) by antioxidants. Thus, increased oxidant production and/or decreased antioxidant function can lead to increased oxidant stress. Increased production of oxidants in diabetes occurs due to high glucose. Brownlee et al, have shown that high glucose activates superoxides production (a form of oxidant) in the mitochondria [9]. According to their hypothesis, the superoxides produced by the mitochondria lead to inhibition of a particular glycolytic enzyme glyceraldehyde 3 phosphate dehydrogenase (GAPDH) causing a build-up of molecules upstream of this enzyme. These molecules are then shunted to other pathways and can activate PKC β, aldose reductase and other pathways.

Increased hyperglycemia derived electron donors from the TCA cycle (NADH and FADH) generates a high mitochondrial membrane potential by pumping protons across the mitochondrial inner membrane. This inhibits electron transport at complex III, increasing the half life of free radical intermediates of coenzyme Q (ubiquinone), which reduce O_2 to superoxide. Another well described source of increased oxidant production is via activation of an enzyme called reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NADPH oxidase generates superoxide by transferring electrons from NADPH inside the cell across the membrane and coupling these to molecular oxygen to produce superoxide anion, a reactive free radical [10].

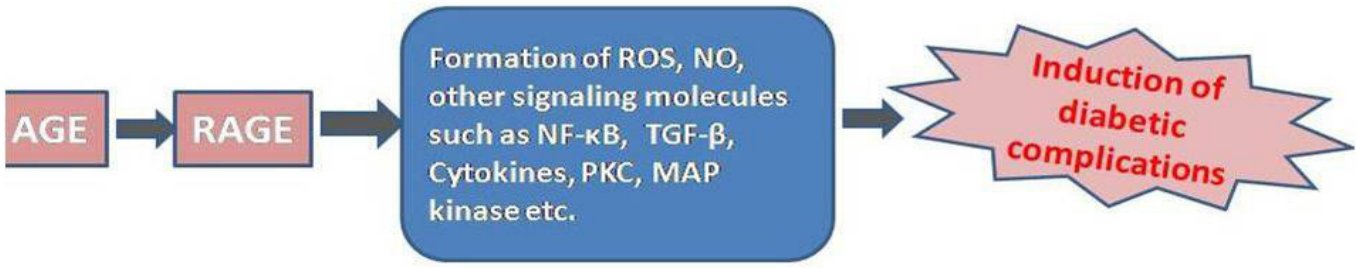


Figure 4: Hyperglycemia and oxidative stress.

The mechanisms described above are the causal factor in the development and progression of diabetic complications by increased OS development. Under hyperglycemic conditions, the factors that stimulate the above mentioned enzyme remained to be defined. AGEs may be one of an important factor which leads to increased NADPH oxidase activity and thereby enhance the OS. NADPH oxidase is a critical component of macrophages and neutrophils and is also found in many other cell types. NADPH oxidases are multi-subunit, membrane associated proteins that catalyze electrons reduction of oxygen using NADPH as an electron donor [11]. Diabetic patients exposed to high level of OS, which play an important role in pathogenesis

of diabetes associated complications. Increasing evidence in both experimental and clinical studies suggested that OS plays an important role in the pathogenesis of diabetes mellitus [12, 13].Oxidative stress has also been strongly implicated in the development and progression of vascular complications [14]. Chronic exposure of biomolecules like lipids, proteins and DNA to higher level of ROS leads to peroxidation and oxidation reaction that results in protein carbonyl (PCO) formation, oxidants of thiol (T-SH) groups, advanced oxidation protein products (AOPP) generation, lipid peroxidation and DNA damage. These OS markers have been shown to be enhanced significantly in diabetic patients [13].

Serum malondialdehyde (MDA) level is a sensitive marker of lipid peroxidation that is a useful measure of OS status. MDA is a decomposition product of peroxidised polyunsaturated fatty acid. Lipid peroxidation was estimated by measuring the level of MDA through thiobarbituric acid reaction [15]. In our previous report, we observed higher serum MDA level in patients with diabetes mellitus compared with healthy controls and significantly more elevated in patients having vascular complications compared with T2DM patients without vascular complications showing enhanced OS in diabetic patients [16]. Cakatay et al, has reported that plasma level of MDA in diabetic patients with poor glycemic control may contribute to the development of diabetic complications [17]. Various studies have also reported high levels of lipid peroxidation in diabetic patients [16-19]. Lipid peroxidation of several structures, a consequence of increased oxygen free radicals, is thought to play an important role in the development of vascular complications in diabetes [20]. MDA is a major player in low density lipoprotein (LDL) modification and is a product of the peroxidation of arachidonic, eicosapentaenoic and docosahexaneoic acids [21]. Oxidised-LDL (ox-LDL) results from the interaction between aldehydes such as MDA and lysine residue in apoB-100 of LDL [22]. The pathologic effects of ox-LDL plays an important role in the induction of diabetic complications.

Another important marker of protein oxidation AOPP has begun to attract the attention of various investigations. Advanced oxidation protein products have been described by Witko-Sarsat et al for the first time [23]. They are formed during OS by the action of chlorinated oxidants, mainly hypochlorous acid and chloramines (produced by yellow peroxidaes in activated neutrophils). AOPP are defined as dityrosine-containing cross-linked protein products and are considered as a reliable marker to estimate the degree of protein oxidation [23]. Protein oxidation may represent an important mechanism in the onset of complications in patients with diabetes. Oxidative modification of proteins that give rise to dityrosine and carbonyl groups generally cause loss of catalytic or structural function in the affected proteins. It is likely the level of oxidized proteins observed during diabetes may have serious deleterious effects in the development of vascular complications. In our previous study, we reported an elevated level of PCO and AOPP in diabetic patients having vascular complications [16]. Various studies also observed elevated levels of these protein oxidation markers in diabetic subjects [24-26].

The mechanism how AGEs induce OS in diabetes is not clearly understood. AGEs can mediate their effect via specific receptors, such as receptors for AGEs (RAGE). AGE-RAGE interaction activates multiple signals such as NADPH oxidase, p21RAS, NF-kB, MAP kinase, TGF-β, vascular adhesion molecules, etc. This transcribes number of pro-inflammatory genes and subsequently elicits vascular inflammation, over expression of endothelial growth factor, impaired fibrinolytic affinity, platelet aggregation, angiogenesis and thrombosis, thereby playing a central role in the pathogenesis of vascular complications in diabetes by enhancing the OS development [27,28].

2. Biochemistry of Formation of Advanced Glycation End Products

Hyperglycemia associated with diabetes mellitus stimulates non-enzymatic reaction between the free amino groups of proteins and carbonyl groups of reducing sugars or other carbonyl compounds leading to enhanced formation of AGEs, also known as the Maillard reaction [29-31]. Advanced glycation end product formation is a complicated molecular process involving multistep reaction. At an early stage, glucose (or other reducing sugars such as fructose, pentose, galactose, mannose, xylulose) reacts with a free amino group of biological amines to form an unstable compound, the Schiff base which undergoes a rearrangement to a more stable product known as Amadori product [32]. In an intermediate stage, the Amadori product degrades to a variety of reactive dicarbonyl compounds such as glyoxal, methylglyoxal, and deoxyglucosones via dehydration, oxidation and other chemical reactions. In the late stage of glycation, non-reversible compounds called AGEs are formed through oxidation, dehydration and cyclization reactions (Fig 5). The AGEs are yellow-brown, fluorescent and insoluble adducts that accumulate on long-lived proteins, thus impair their physiological functions [33]. AGEs-modified proteins lose their specific functions and undergo accelerated degradation to free AGEs such as 2-(2-Furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI), imidazolone, N-ε-carboxy-methyl-lysine (CML), N-ε-carboxy-ethyl-lysine (CEL), glyoxal-lysine dimmer (GOLD), methyl-glyoxal-lysine dimer (MOLD), and others. In addition, AGEs can also act as cross-linkers between proteins, resulting in the production of proteins-resistant aggregates.

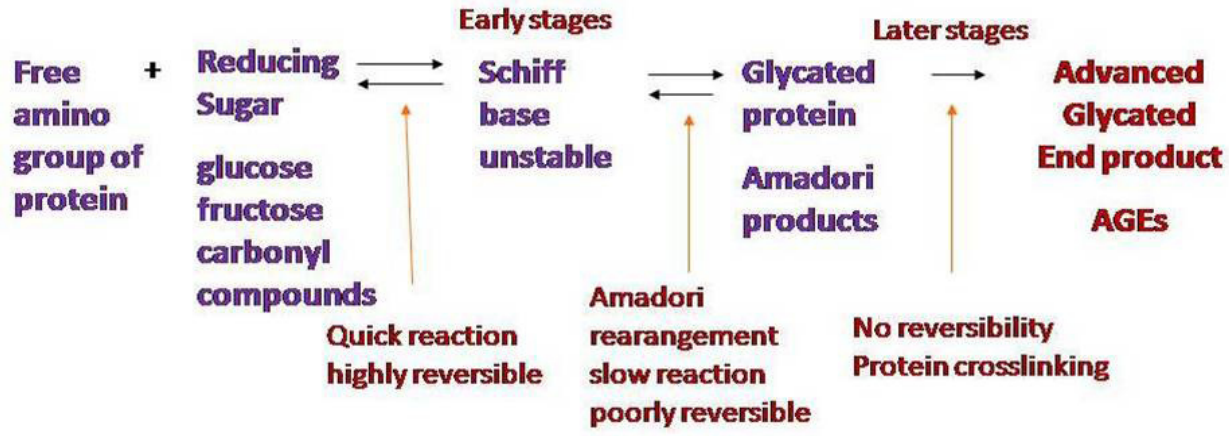


Figure 5: Formation of advanced glycation end products.

In the Maillard reaction, formation of reactive intermediate products during Amadori rearrangement is very important. These compounds are known as α-dicarbonyls or oxoaldehydes such as 3-deoxyglucosone, methylglyoxal (an intermediate product of Maillard reaction) [7]. Furthermore, humans are also exposed to exogenous AGEs, which are ingested with food. Over a dozen AGEs have been detected in tissues and can be divided into three categories: 1. Fluorescent cross-linking AGEs such as pentosidine and crossline. 2. Non-fluorescent cross-linking AGEs such as imidazolium dilysine cross-links, alkyl formyl glycosyl pyrrole (AFGP) cross-links and arginine-lysine imidazole (ALI) cross-links. 3. Non-cross-linking AGEs such as pyrraline and N-carboxymethyllysine (CML) [34].

The other well studied mechanism for the formation of AGEs is the polyol pathway, where glucose is converted into sorbitol by the enzyme aldolase reductase and then to fructose by the action of sorbitol dehydrogenase [35, 36]. Fructose metabolism as fructose 3-phosphate, then is converted into α-oxaldehydes and interacts with monoacids to form AGEs. Thus, at least three pathways are responsible for AGEs formation including Malliard reaction, oxidation of glucose and peroxidation of lipids and finally through polyol pathway. The serum AGEs level was determined spectrofluorometrically at emission maximum (440 nm) upon excitation at 350 nm [26]. Briefly, serum was diluted 1:50 with phosphate buffer saline (PBS) (pH=7.4) and fluorescence intensity was expressed in arbitrary units (AU). Total serum AGEs were also determined by ELISA using commercial kits. Previously, we reported higher levels of circulating AGEs in patients with micro and/or macro-vascular complications indicating that higher the serum AGEs level, higher the likelihood of development of vascular complication of diabetes [16,37,38]. Earlier gradual increase in serum AGEs level have been reported with the severity of atherosclerosis in diabetic patients [39,40]. Kalusova et al determined AGEs spectrofluorometrically and found AGEs were about 23% higher in diabetic patients compared to healthy individuals [26]. In recent studies, AGEs level has been suggested to act as a predictor of CVD mortality and diabetic nephropathy [39-43].

2.1. Metabolism of advanced glycation end products

Once the AGEs are formed, they interact with their receptors namely: AGE-R1, AGE-R2, AGE-R3 and RAGE.

2.1.1. AGE-R1 (oligosaccharyltransferase-48)

AGE-R1 is a cell surface associated receptor that opposes excessive ROS generation by AGEs. AGE-R1 is linked to the endocytosis and removal of AGEs [44] and to the suppression of MAPK and NF-kB activity, via inhibition of AGE-induced ROS generation [45]. Thus, AGE-R1 appears to control the activation of distinct cellular pathways and protects against

vascular disease promoted by oxidants [46]. Interruption of AGE-R1 dependent uptake of AGEs and subsequent degradation is associated with accelerated glomerular renal pathology in the spontaneous non-obese diabetic strains of mice [47]. AGE-R1 may be suppressed or saturated in circumstances of sustained AGE-induced OS when RAGE is up-regulated and AGE-R1 to RAGE ratio is negative. Diminished expression of AGE-R1 in circulating mononuclear cells and a corresponding elevation in serum AGE levels are seen in human subjects with severe diabetic complications [47].

2.1.2. AGE-R2 (80K-H phosphoprotein)

AGE-R2 is an 80 to 90kD protein involved in the intracellular signaling of various receptors like fibroblast growth factor receptor. AGE-R2 contains a tyrosine phosphorylated section in the plasma membrane of the cell [48].

2.1.3. AGE-R3 (galectin-3)

AGE-R3 belongs to lectin family of carbohydrate binding protein. AGE-R3 is up-regulated in hyperglycemia after exposure to AGEs. Galectin-3 knockout mice have developed accelerated glomerulopathy in response to diabetes, with increased renal glomerular AGE accumulation and diminished scavenger receptor expression [49].

2.1.4. Receptor for advanced glycation end products (RAGE)

RAGE is the best characterized and the most studied receptor for AGEs [50]. The main receptor which is responsible for AGEs related diabetic complications is RAGE which appears to activate a stress response leading to inflammation and cellular dysfunction. AGEs bind to specific receptor RAGE which is expressed in many of the cell types which includes endothelium, monocytes/macrophages, T-lymphocytes, neuronal cells and glomerular epithelial (podocyte) cells [51-54]. RAGE is highly expressed at the mRNA and protein levels in early developmental stages under normal physiological conditions [55]. RAGE expression occurs in most tissues, including the heart, liver, brain and kidney [56-58]. Since its isolation in 1992, a growing body of scientific evidence has demonstrated a role for RAGE in the pathogenesis of diabetes and its vascular complications [59].

2.1.5. Molecular structure of RAGE

RAGE is a 45kD transmembrane receptor of immunoglobin super family and is composed of 404 amino acid [50]. RAGE consists of three distinct domains as shown in Fig.6

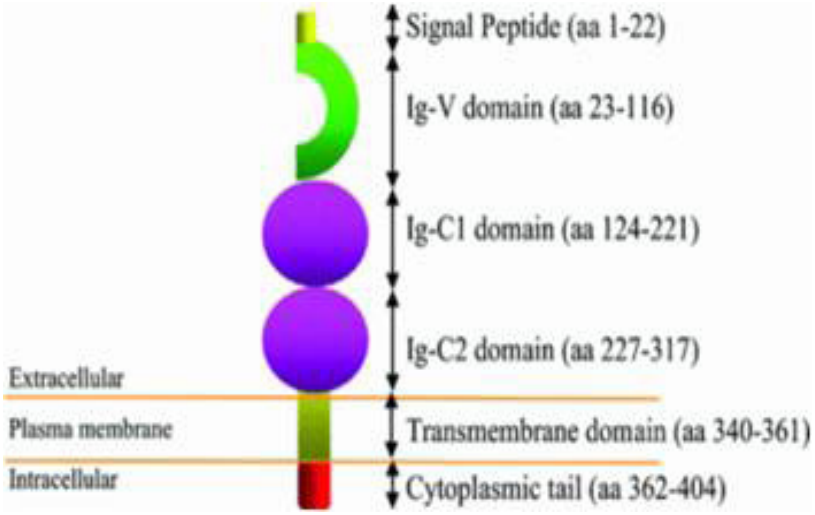


Figure 6: Molecular structure of RAGE (Adapted from kalea et al. [53])

1. Extracellular domain (amino acids 1-339): This extracellular component has three immunoglobulin-like (Ig) domains. The N-terminal Ig domain is assigned to the V-set of Ig-like molecules and is known as the V domain of RAGE. The other two Ig domains are part of the C1 and C2 set. The N-terminal V domain is located far away from the plasma membrane, but the C2 domain lies close to the membrane. V and C1 domains can be joined together to form an elongated structure. V and C1 domains can be fixed and become the VC1 domain, which can connect to the C2 domain by several amino acids that have no secondary structure, which allows the VC1 and C2 domains to link. NMR spectroscopy studies have shown that VC1 moves as a single unit and can combine with the C2 domain.

The V domain consist a large amount of arginine and lysine, which carry positive charge at neutral pH. RAGE V domain has more arginine and lysine than the V-set of Ig domains. Arginine and lysine residues form large positively charged patches on the surface of the V and C1 domains. Meanwhile, C2 domain has mainly acidic residues on its surface and is negatively charged. Because the two domains are oppositely charged, the extracellular component of RAGE is subdivided. This subdivision is reflected in the ligand binding properties of the different domains since ligands do not bind to C2 due to charge repulsion. Most ligands tend to bind to the V domain or the VC1 domain since ligands are negatively charged. There is only one case where a ligand has bound to the C2 domain. Nonetheless, charge-charge interactions are important for the formation of the receptor-ligand complex and suggested that the positively charged ligand-binding domain of the RAGE molecule can recognize certain arrangements of negative charges of ligands and can recognize these as common features for the ligands.

2. Single transmembrane domain (amino acid 340-361): Transmembrane domain is a single hydrophobic helix.

3. Short intracellular cytoplasmic tail (amino acids 362-404): An intracellular domain consists of highly acidic short 42 amino acid cytoplasmic tail, which is essential for RAGE-mediated signaling and overall RAGE function.

Apart from the full length, RAGE also available as soluble circulating isoform including sRAGE1/2/3, esRAGE (endogeneous soluble RAGE) and hRAGEsec (human RAGE secreted). A number of mechanisms have been reported that lead to the production of soluble proteins, alternative splicing of the mRNA to remove the transmembrane domain and the proteolytical cleavage from the cell surface. Various studies of RAGE have shown that sRAGE can be formed by both alternative splicing and proteolytic cleavage [60-62].

3. Role of RAGE Genetic Variants And Its Expression In Diabetes And Its Complications

Type 2 diabetes mellitus is genetically heterogenous disease, caused by interaction between genetic and environmental factors [63]. As described earlier, prevalence of T2DM varies with geographic regions and ethnicity of the population and AGE-RAGE interaction play a significant role. The up-regulation and pathogenic effects of RAGE in diabetic vascular complication as well as multiple genetic variants identified for RAGE, suggests a significant role of RAGE as an important contributing mechanism in diabetes and its complications. The gene encoding RAGE is located on chromosome 6 in major histocompatibility complex, a region of a genome containing number of inflammatory genes [64]. Genetic studies have identified approximately 30 polymorphisms in the RAGE gene at exons, introns and in the 5' flanking region as shown in Fig 7 [65]. Sequence variation in the RAGE gene has been studied and a relatively large number of single nucleotide polymorphisms (SNPs) in the coding and non-coding region of the RAGE gene have been identified recently. The functional impact of several of them on the transcriptional activity, ligand binding or intermediate phenotype has been described.

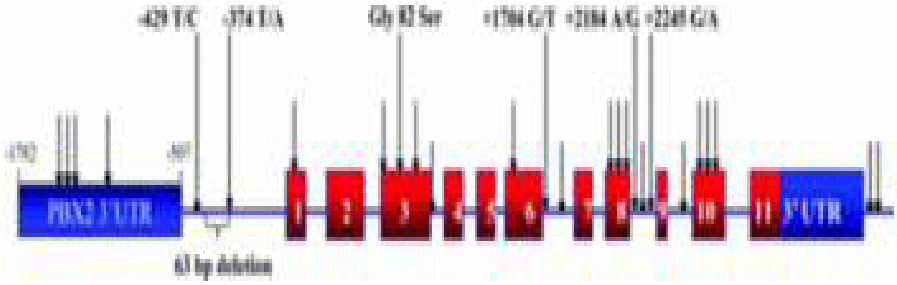


Figure 7: SNP map for RAGE gene. (Adapted from Hudson et al. [65])

3.1. Promoter region polymorphism of RAGE gene

The up-regulation of RAGE gene expression is a hallmark of vascular disease and therefore genetic variation affecting RAGE m-RNA or protein levels may therefore an important disease marker. The RAGE gene is regulated by a promoter region (1.5 KB) up-stream of the transcription start site and within this region, numerous polymorphisms have been identified including -374T/A and -429T/C substitution [65]. Recent studies have revealed that -374 T/A (rs 1800624) and -429T/C (rs 1800625) polymorphisms in the promoter region considered

as an important genetic variation as targets for association studies due to its marked effect on transcriptional activity which may alter AGE-RAGE interaction leading to an altered signaling cascade [66-68].

Falcone et al has reported that conversion of T to A substitution of -374 T/A polymorphism results in repression of receptor expression inefficiency in RAGE cellular signaling. In our previous study, we reported protective nature of -374A mutant allele towards macrovascular complications in diabetic subjects [69, 70]. Similar observations were found in case control studies which revealed a strong link between an individual's possessions of the -374A allele of AA genotype and protection against vascular disease. This includes decreasing risk of cardiocerebral vascular disease [71-74]. A decrease restenosis of the angioplasty [75], decrease in the number of affected vessels [76,77], a decrease mortality after myocardial infarction [78]. Reduce risk of coronary artery disease for -374A allele has reported in African – Brazilian and Caucasian-Brazilian population independent of the risk factor associated with this complication [73,77]. In Caucasian populations Zee et al has reported that patients carry -374A variant has reduced risk of ischemic stroke [72]. However, no association has been reported with diabetic retinopathy [79,80]. A meta analysis report has also reaffirmed the protective nature of -374A allele towards development of macrovascular complications in DM patients [81].

Proximal to the −374T/A polymorphism, −429T/C is another polymorphic variant. Although this variant was shown to increase transcriptional levels in various studies, unlike the −374T/A SNP. In our recent study, we reported -429C mutant allele was associated with the development of macrovascular complications in T2DM subjects in a highly significant manner ($p < 0.001$) [70]. However, in Chinese and Slovene population, no association has been reported [82,83]. In contrast, another study reported increased risk of diabetic retinopathy among patients with the TC or CC genotypes [68]. Not many studies are available on -429T/C promoter polymorphism in Indian population.

The dissimilar association of two promoter polymorphisms i.e. -374T/A and -429T/C reveals an interesting outcome. The protective nature of -374T/A polymorphism towards macrovascular complication, while -429T/C showed significant association towards the development of macrovascular complication in diabetic patients. This opposite phenotypic characteristics of these two promoter region polymorphisms may be attributed to their dissimilarity in association to diabetic complications and also due to strong linkage disequilibrium. -374T/A polymorphism has been shown to cause repression of RAGE gene expression [68]. The lesser the RAGE gene expression, lesser will be its binding with ligand and consequent lesser signal transduction for pro-coagulant or pro-thrombotic gene and thereby imparting protection towards vasculature. On the other hand -429T/C promoter polymorphism has been shown to increase RAGE gene transcript [76]. The enhanced availability of RAGE augments AGE binding and induce downstream signaling resulting in release of proinflammatory cytokines and

adhesion protein that favors thrombosis and eventually capillary leakage and occlusion.

3.2. Coding change polymorphisms in the RAGE gene

The G82S polymorphism was one of important identified naturally occurring polymorphism of the RAGE gene at position 82 proximal to an N-glycosylation site (position 81) is involved in ligand binding and downstream signaling has also attracted considerable interest and therefore strongly suggests that this variant may affect RAGE function [65,84]. This polymorphism is the only frequent coding-change polymorphism in the RAGE gene, with all other polymorphisms identified to change the amino acid sequence occurring in less than 1% of subjects [65]. Most recently, Xie et al. [85] investigated the protein structural changes of this variant and revealed that the Ser82 substitution lead to local structural changes in the V-domain, and also it imparted more widespread overall tertiary structural alterations in the extracellular domain. In our previous study, we observed a significant association of Ser82 mutant allele with microvascular complications in T2DM patients [70]. Similar to our finding, the mutant allele (Ser82) has been shown to be associated with the development of microvascular complications in Chinese, Caucasian and North Indian population [86-89]. A meta analysis report has also reaffirmed the significant association of Gly82Ser polymorphism towards development of diabetic retinopathy among Asian populations [90]. Although, various studies found no association between Gly82Ser polymorphism and diabetic complications in various other populations such as Malaysian, Brazilian and Japanese [91-93]. Mutant allele (Ser82) of G82S RAGE displays enhanced ligand binding and downstream signaling which leads to microvascular complications. How RAGE signaling induces microvascular complications in T2DM subjects has not been fully elucidated. It was reported that NF-κB mediated enhanced expression of pro-inflammatory and pro-fibrotic cytokine TGF-β is responsible for retinal and renal damage, characteristics of microvascular complications [94,95].

The other functional variant identified in the RAGE gene was a 63 bp deletion which spans from −407 to −345 of the promoter (transcriptional) region. Due to its incidence being <1% in populations the use of this variant as a genetic marker for the disease is limited to date [67]. Studies of other RAGE gene promoter variants in the 5' flanking region make difficult due to the overlapping 3′-UTR of the PBX2 gene on chromosome 3 [94]. In essence, this means any variant amplified within this region is in fact amplifying both chromosome 6 and 3 and therefore renders it very difficult to genotype specific variants. The other possible genetic variation in the RAGE gene occurs within the intronic regions, which may affect RAGE mRNA splicing and therefore alter the ratio of splice variant and hence the levels of full-length and sRAGE. Although no variants exist proximal to or within exon/intron boundaries, a number of variants have been identified within introns 7 and 8 [94]. These include the +1704G>T, +2184A>G and +2245G>A which were first identified in a Czech population and demonstrat-

ed to be associated with diabetic microvascular dermatitis [94]. Subsequent studies by these researchers demonstrated an association of the +1704G>T and +2184A>G with antioxidant status in Type 2 diabetes, but not with proliferative retinopathy [89]. However, limited studies of only the +1704G>T have been performed in other populations [89,93]. Further studies in large numbers of subjects are required to thoroughly investigate these variants.

4. Therapeutic Intervention of AGEs

Inhibition of AGEs formation and attenuating the AGEs-mediated effects may be considered as ideal candidates for pharmaceutical intervention in the amelioration of diabetic vascular complications. Therapies against the AGEs mediated effect can through diverse pathways, like inhibiting the production of Amadori products, decreasing AGE-RAGE interaction, detoxifying dicarbonyl intermediates and interrupting biochemical pathways that impact on AGEs level. Since OS plays an important role in the development of vascular complications, antioxidants shows beneficial effects on AGE- mediated vascular complications through suppression of intracellular ROS generation.

4.1. Aminoguanidine

Aminoguanidine (Pimagedine,) is a pharmacological inhibitor of the advanced glcosylation pathway and it also reacts with many biological molecules, including pyridoxal phosphate, pyruvate, glucose, MDA, and others [30, 96]. Aminoguanidine is a therapeutic agent for prevention for AGEs formation. It reacts rapidly with alpha, beta-dicarbonyl compounds such as methylglyoxal, glyoxal and 3-deoxyglucosone to prevent the formation of AGEs [97]. Inhibition of disease mechanisms, particularly vascular complications in diabetes, by aminoguanidine has provided evidence that accumulation of AGEs is a risk factor for disease progression [30]. Aminoguanidine has other pharmacological activities, inhibition of nitric oxide synthase and semicarbazide-sensitive amine oxidase, at pharmacological concentration achieved in vivo for which controls are required in anti-glycation studies [98]. Use of the high concentration of aminoguanidine in-vitro brings these reactions and related effects into play. Aminoguanidine prevents the formation of fluorescent advanced non-enzymatic glycosylation products and of glucose derived collagen cross links in-vitro. It appears that the primary mechanism by which aminoguanidine inhibits the formation of advanced glycosylation end products is by reacting with Amadori derived fragmentation products such as 3-deoxyglucosone [99].

4.2. N-acetylcysteine

N-acetyl-L-cysteine (NAC), a thiol containing acetylated form of the amino acid L-cysteine that functions as a precursor of glutathione synthesis [100]. Glutathione (GSH) is an

important thiol involved in cellular detoxification. GSH plays a physiological role in maintaining the body homeostasis and in protecting cells against oxidants, toxicants, DNA damaging agents, and carcinogens of either exogenous or endogenous source [101]. The presence of sulfhydryl groups in NAC also enables the neutralization of free radicals. It is a powerful scavenger of HOCl and is capable of reducing HO and H_2O_2 and act as a powerful antioxidant [102]. NAC occurs in two forms L and D, only L-NAC is active; L-NAC is metabolized to cysteine and then GSH, but D-NAC is not. NAC acts outside the cell to reduce cystine to cysteine, which can be transported into the cell 10 times faster than cystine and further used in the biosynthesis of GSH. By facilitating GSH biosynthesis, NAC serves an indirect anti-oxidant role where it can enhance glutathione-S-transferase activity; supply GSH for glutathione peroxidase catalyzed detoxification of peroxides [103]. This compound is sold as a dietary supplement commonly claiming anti-oxidant and liver protecting effects. It is used as a cough medicine because it breaks disulfide bonds in mucus and liquefies it, making it easier to cough up. It is useful in thinning the abnormally thick mucus in cystic and pulmonary fibrosis patients [104].

NAC has found to protect the beta cells in culture and in vivo from “glucose toxicity” preserving insulin synthesis and secretion [105]. Gibson et al suggested that NAC reduce thrombotic propensity in T2DM patients by increasing platelet anti-oxidant status and GSH synthesis, thereby lowering platelet derived ROS [106]. NAC helps to increase nitroglycerin activity, thereby, potentiates the coronary dilating and anti-platelet effects of nitroglycerin and limits the development of hemodynamic tolerance to nitroglycerin. AGE-RAGE mediated ROS generation induces mesangial cell hypertrophy, and fibronectin synthesis has been reported to be inhibited by NAC [107].

4.3. Resveratrol

Resveratrol (3,5,4 trihydroxy-trans-stillbene) is a stilbenoid, a type of natural phenol, and phytoalexin produce naturally by several plants. Resveratrol is a member of a group of plant compounds called polyphenols. These compounds are thought to have anti-oxidant properties, protecting the body against the kind of damage linked to increased risk for conditions such as cancer and heart disease. Beneficial effects of resveratrol including telomere lengthening, telomerase activity enhancement, anti-inflammatory and blood sugar lowering have been reported in mouse and rat model. Reseveratrol gets extensively metabolized in the body. Liver and gut is the major site of metabolism. [108]

Resveratrol has been shown to inhibit platelet activation and aggregation, suppress TNF-induced activation of nuclear transcription factors [109], inhibit the generation of reactive oxygen species by human polymorphonuclear leukocytes [110]. Resveratrol has also been reported to reduce the risk of cardiovascular and tumoral disease by acting on the mechanisms

that regulate the expression of growth factors and cytokines such as transcription factor NF-kB [111]. Resveratrol has been reported to afford cardiovascular protection and to reduce atherosclerosis by various mechanisms. These include modulation of lipid turnover, production of eicosanoids, oxidation of lipoproteins and reduction of platelet adhesion [112]. Thus, resveratrol may be considered as an effective therapeutic agent for the treatment of diabetes mellitus and its associated complications.

4.4. Curcumin

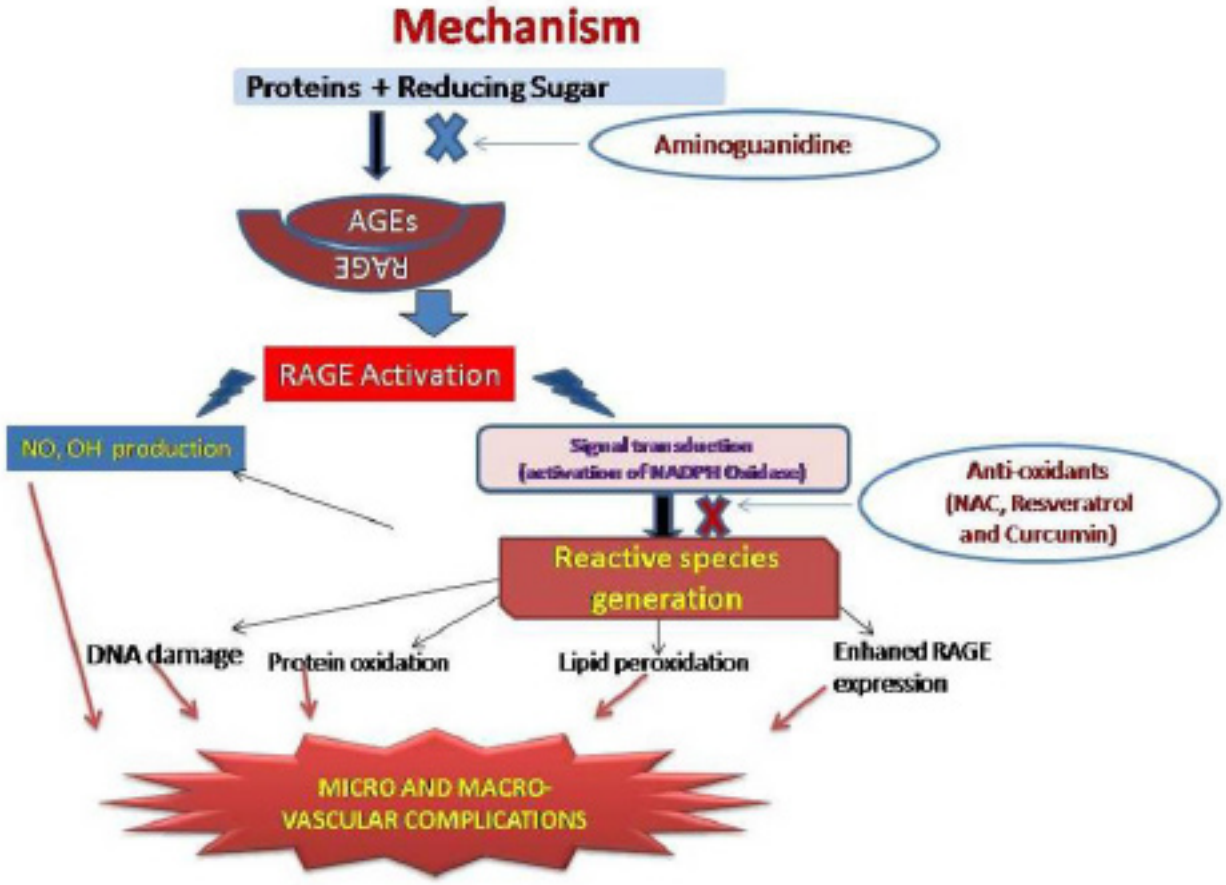
Curcumin is the principal curcuminoid extracted from the rhizomes of the plant Curcuma longa (popular Indian spice turmeric), a member of ginger family found in south and southeast tropical Asia [113]. The curcuminoids are polyphenols and are responsible for the yellow color of turmeric. Curcumin can exist in at least two tautomeric forms, keto and Enol. The Enol form is more energetically stable in the solid phase and in solution. Curcumin incorporates several functional groups having molecular formula $C_{21}H_{20}O_6$. Potential factors that limit the bioavailability of curcumin include poor absorption, rapid metabolism and rapid systemic elimination. Numerous approaches to increasing curcumin bioavailability have been explored, including the use of adjuvants [114].

Curcumin has shown diverse and versatile beneficial effects, including anti-inflammatory, anti-oxidative, anti-viral, anti-hypercholestermic, anti-infective and anti-carcinogenic effects [115]. This polyphenol has been shown to reduce risk of cancer, heart disease, Alzheimer's disease, and diabetes mellitus [116]. Curcumin supplementation improved diabetes-induced endothelial dysfunction through lowering plasma glucose levels, decreasing superoxide production and inhibiting protein kinase C activation [117]. The preventive effect of curcumin on the level of AGEs and cross-linking of collagen in diabetic rats has been reported with respect to prevention of AGE-induced complications in diabetes mellitus [118]. Xu et al. has reported that curcumin inhibited the proliferation of activated hepatic stellate cells and this has been mediated by curcumin induction of gene expression of PPARg and PPARg activation [119]. Okamoto et al has also been reported the preventive effects of curcumin on AGE-induced increase in NF-kB and AP-1 activity, vascular endothelial growth factor m-RNA up-regulation and the resultant increase in DNA synthesis in microvascular endothelial cells [120].

5. Summary

This chapter summarizes that AGEs formation appears to be enhanced under hyperglycemic condition. Increased glycation of plasma protein and its accumulation plays an important role in the pathogenesis of diabetic vascular complications. Up-regulation and pathogenic effect of AGEs receptor, i.e. RAGE, expression and its genetic variant plays an important role in the pathogenesis of diabetes and its vascular complications, the molecular mechanism of

activation of RAGE needs to be investigated. The possibility of reducing glycation of protein or circulating AGEs or blocking RAGE is an approachable target of delaying or preventing the onset of diabetic complications. Various compounds are under investigation for their possible therapeutic intervention. Finally, the use of AGEs as biomarkers/predictors of diabetic complications may be helpful to reduce health problems in diabetic patients.



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Advances in Biochemistry & Applications in Medicine

Chapter 2

Functional Nanocrystals: Towards Biocompatibility, Nontoxicity and Biospecificity

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Abstract

The tunable surface chemistries have dramatically increased by expanding the structural arrangements of nanocrystals with many different strategies. Novel synthesis of functional colloidal nanocrystals has been developed over the past decade enabling the production of highly uniform and stable nanoparticles with important photonic characteristics. In this chapter, we will specifically comment on methodologies used in QDs syntheses that make nanocrystals highly biocompatible with important applications as luminescent probes. In addition, we will also discuss a new category of QDs named magic sized quantum dots (MSQDs), which present intense and broad luminescence rangeand greater size stability, turning them special tools for multiple biological applications in diagnosis, monitoring and therapy. Special emphasis will be placed on,safety, biocompatibility and biospecificity of nanomaterials, which are of great concern as new techniques and novel nanocrystals emerge.

Keywords: nanocrystals; magic sized quantum dots; biocompatibility; genotoxicity; specific probe

1. Introduction

Quantum dots (QDs) of cadmium chalcogenides (CdSe, CdS and CdTe) have been used in several biological applications, since both absorption and emission spectra can be controlled as a function of size and shape [1-3]. As biological markers, QDs present several advantages over traditional organic fluorophores, such as long fluorescence lifetime(around 100 times greater) that allows to distinguish it from the background signalsobserved as autofluorescence-withshorter fluorescence absorption lifetime [4], greater photo-resistance and chroma-degradation, and a highlytunable fluorescence intensity [4,5]. However, QDs cytotoxicity is still a highly important subject due to its unpredicted behavior in the environment and within biological systems. We will specifically discuss, in this chapter, QDs syntheses methodologies, structural and physical characterizations, surface functionalization and bioconjugation, safety, biocompatibility and their applications

Zinc oxide (ZnO) and titanium dioxide (TiO_2) nanocrystals (NCs) have been widely used in sunscreens; therefore, biocompatibility and toxicity are inherently important subjects to investigate. ZnO is a group II-VI semiconductor with a hexagonal wurtzite crystal structure, with a broad energy band (3.37 eV), high bond energy (60 meV), and high thermal and mechanical stability at room temperature [6]. Furthermore, ZnO nanocrystals have antibacterial properties [7], and it has been added to several materials, including cotton fabrics, rubber and food packaging [8].

TiO_2 is semiconductor with bandgap around 3.0 eV (rutile) or 3.2 eV (anatase), with high photochemical stability [9]. When the size of the NCs is greatly reduced, the surface volume ratio increases and the quantum confinement effects may appear. In addition to size and shape, the crystalline phases of TiO_2 NCs also enable changes in physical properties. TiO_2 is a polymorphic material with three allotropic forms, namely anatase, brookite and rutile, with tetragonal ($I4_1/amd$), orthorhombic (Pbca) and tetragonal ($P4_2/mnm$) forms, respectively [10]. The anatase phase of TiO_2 NCs presents higher electron mobility, low dielectric constant, lower density, lower capacity to adsorb oxygen and higher degree of hydroxylation. The rutile phase has the highest thermodynamic stability at room temperature and pressure, but with higher levels of defects, besides being easier to be reduced. The brookite is the least studied crystalline phase due to the difficulties of production of a pure phase. This phase is often observed as a by-product when the precipitation is carried out in an acidic medium.

In view of these broad characteristics the systematic study of biocompatibility and genotoxicity in function of the size and crystalline phase of ZnO and TiO_2 NCs will also be approached in this chapter

2. Nanocrystals and quantum dots

The theory of crystal formation in solution is not a very simple matter and involves the analyses of several parameters used in the synthesis process. This section will cover an overview of crystal nucleation and growth processes.

The crystal growth occurs due to the crystallization of a homogeneous phase that begins and extends progressively from discrete centers distributed throughout the material. Thus, crystal formation occurs by sequential and periodic ordering of atoms in a standard structure and their growth due to the attachment of species present in the solution capable of reacting at the growth site, producing elements that will be part of the crystal structure. The crystal grows in the solution in order to decrease the free energy in the system as a result of two factors: achieving an orderly distribution of particles with maximum compensation of the chemical bonds and the mobility of the particles [11].

Figure 1 represents in (a) the most convenient energy position for grouping elementary entities that are at point 1, in which the least convenient is point 6. Small numbers indicate (in arbitrary units) the amount of energy released with the grouping of elementary entities in a given position (b) to the process of nutrition of the phases. Thus, nanocrystals are nanometric scale crystals.

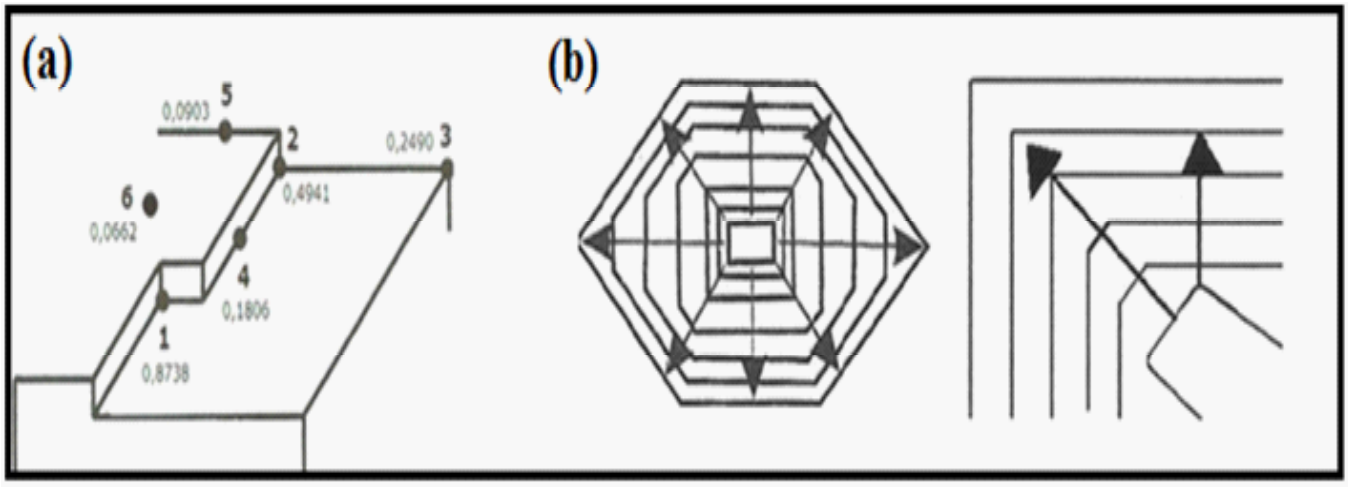

Figure 1.(a) Energy positions in the crystal; (b) Growing face of the crystal [11]

Nanocrystals can exhibit bulk and quantum confinement properties depending of the size and shape. Quantum dots (QDs) are nanocrystals that present quantum confinement in three dimensions. These dimensions must be larger than the lattice parameter and less or equal than Bohr radius of the corresponding bulk material. The Bohr radius of the exciton (r_{Bexc}) is the distance of the electron-hole pair, which is inversely proportional to the effective mass of the carriers (electrons and holes). The effective mass takes into account the mass of the carrier (electron or hole) plus the interactions between it and the ions of the crystalline lattice of the material [12]. Each material presents an effective mass of its carriers and consequently a given

Bohr radius, so depending on the material there may be different ranges of sizes with quantum confinement properties.

Figure 2 shows a schematic representation of spherical nanocrystals that presents quantum confinement properties (quantum dots) and bulk (without confinement), as well as the energy variation of the exciton with the size of the nanocrystal. When the radius of NCs is larger than the Bohr's exciton (r_{exc}) there is no quantum confinement property (bulk). In nanocrystals with bulk properties, energy levels are close to each other forming bands. Where as in quantum dots, the energy levels are quantized, resembling an artificial atom. Thus, in QDs the energy of the gap is altered with the size, allowing tuning its optical properties. The smaller the QDs size the higher the excitation energy.

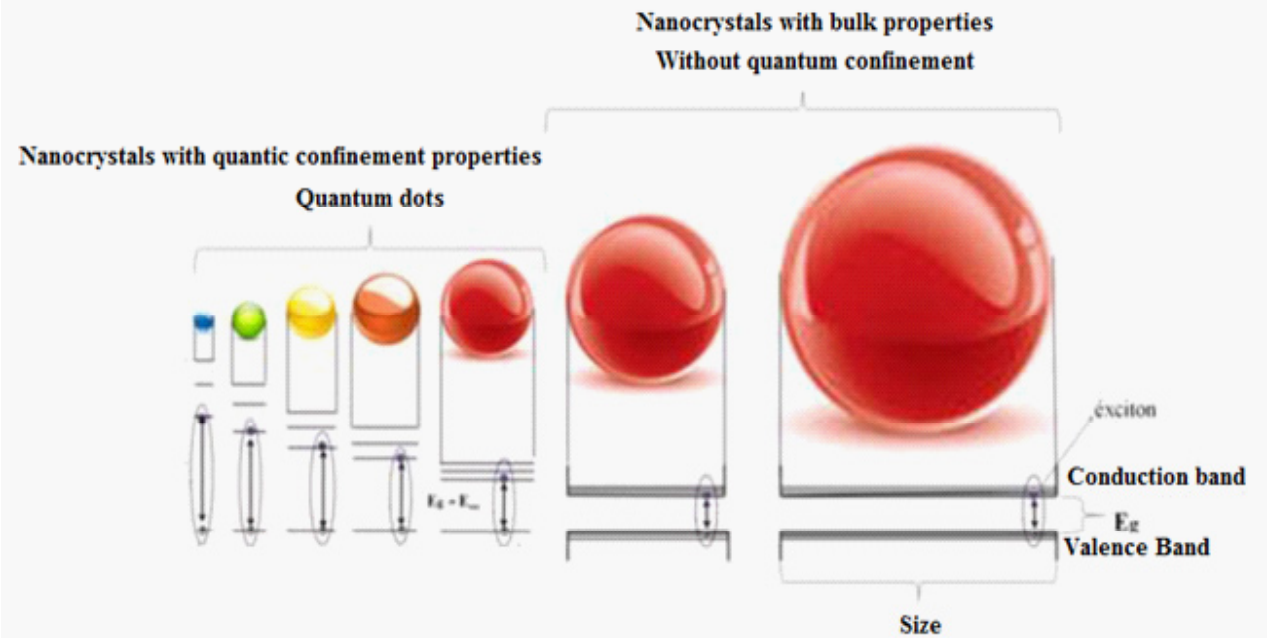

Figure 2: Variation of energy as a function of the size of the nanocrystals with and without quantum confinement properties [13].

The process of luminescence consists of three steps: excitation (creation electron-hole by an external source), thermalization (electrons and holes relax for the conduction band and valence bottom, respectively) and recombination (electrons and holes recombine producing an emission of photons).

Figure 3a illustrates the absorption process of a photon for the creation of the electron-hole followed by termination. The carriers position at the ends of the bands and the radiative recombine, in which a photon emission occurs in nanocrystals with properties of *bulk* and quantum confinement. It is observed that a decrease in size of QDs increases the transition energy between energy bands.

The quantum confinement properties present in QDs can be observed in the absorption and emission spectra, as shown in Figure 3b. In the case of CdSe, the indication of QDs formation is when the absorption band is at wave lengths shorter than the corresponding bulk mat-

erial (CdSe = 708 nm). The redshift between absorption and emission bands occurs due to decreased energy gap caused by the increase in size of QDs. Thus, based on the absorption and emission spectra, it is possible to monitor the growth kinetics.

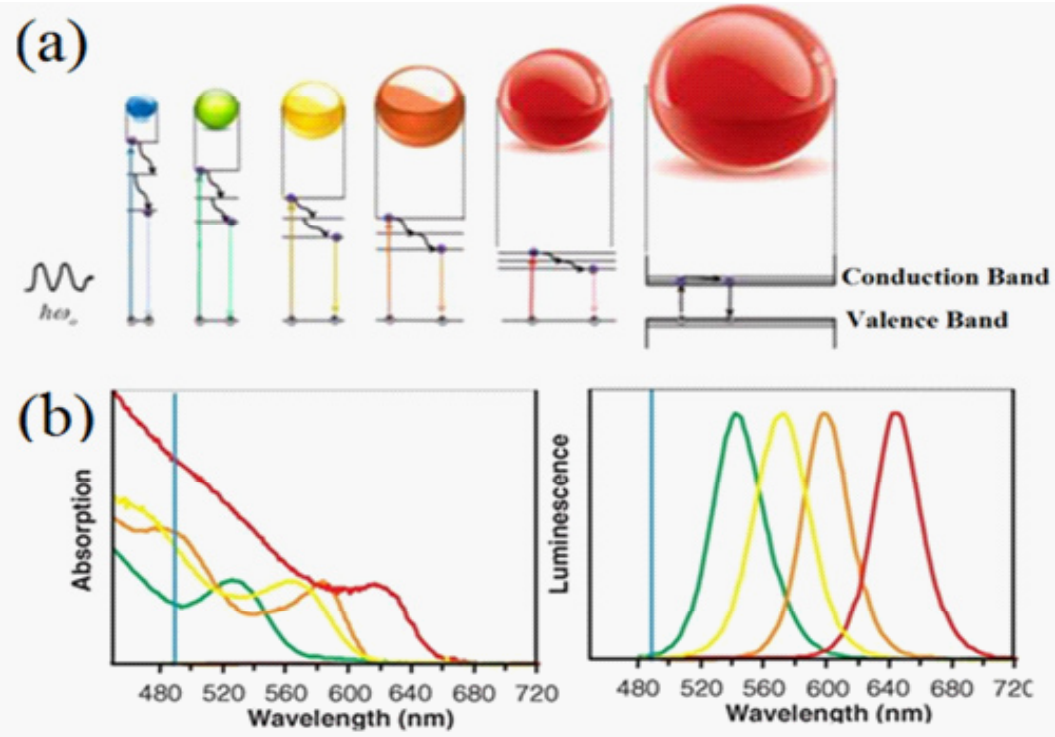

Figure 3: (a) Representative scheme of the absorption and emission process [13];(b) absorption and emission spectra of CdSe QDsof different sizes [14].

Magic-sized quantum dots (MSQDs) are quantum dots with extremely small sizes (<2 nm) and physical properties that are completely different from those presented by conventional QDs [15-17]. They present thermodynamically stable structures, wide luminescence range, great size stability over time, relatively narrow absorption spectra, and/or heterogenic growth [16,18-20]. In order to understand these structures Nguyen, Day and Pachtermade theoretical predictions of various types of CdSe MSQDs structures that are in agreement with the experimental results of the literature [21]. The term magic size is related to a (magic) number of atoms in the structure that makes MSQDs extremely stable. The wide luminescence spectrum occurs because MSQDs have internal atomic defects [20,22,23].

In addition to these characteristics, the MSQDs also present great stability of the luminescence within cells, allowing their use in assays that requires biological tracking and monitoring [24]. Examples of such property are shown by fluorescence microscopy images of CdSe MSQDs in HeLa cells in Figure 4, exposing cells for 24h**(a)** and 36h**(b)**.

3. Surface functionalization and bioconjugation

The functionalization process of QDs consists of coating the surface with organic molecules that carry specific chemical groups, such as carboxylic acid, thiol, amine, maleimide, aldehyde, epoxy, among others [25,26]. Such chemical groups in the QDs surface are used not

only to facilitate their dispersion in biological fluids, but also for conjugation with biological molecules, aiming their use as luminescent probes, as shown in Figure 5.

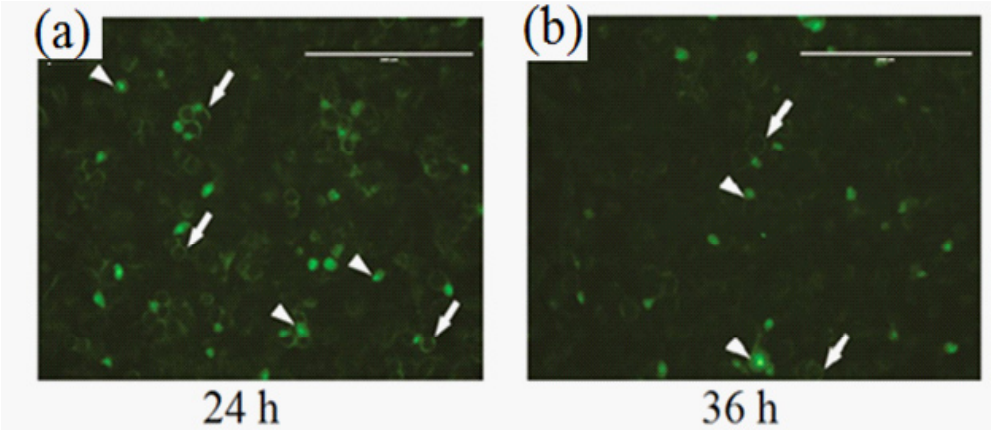

Figura 4: Fluorescence microscopy images of CdSe MSQDs in HeLa cells after (a)24h and(b) 36h [24]

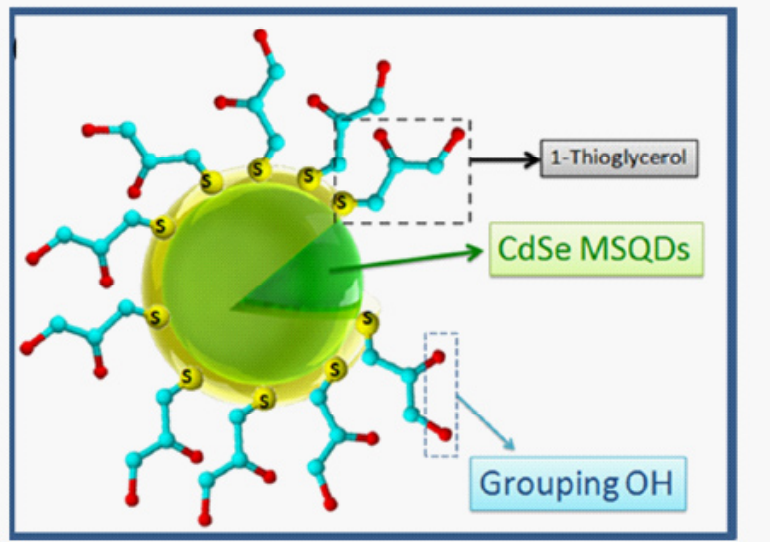

Figure 5: Representation of the functionalization nationalization process in QDs [24]

Another important process is the passivation, which is used to minimize the amount of electronic levels between the valence and conduction bands of the semiconductor, usually as a result of defects caused during the nanocrystal growth. These levels lead to non-radiative pathways by greatly suppressing the luminescence of nanocrystals. Therefore, after performing the passivation procedure, the defect levels decrease. This method resumes covering the core of the nanocrystal with an organic material or a semiconductor with energy gap larger than that of the nucleus as shown Figure 6. Therefore, this procedure is widely used in the synthesis of nanocrystals [27-29].

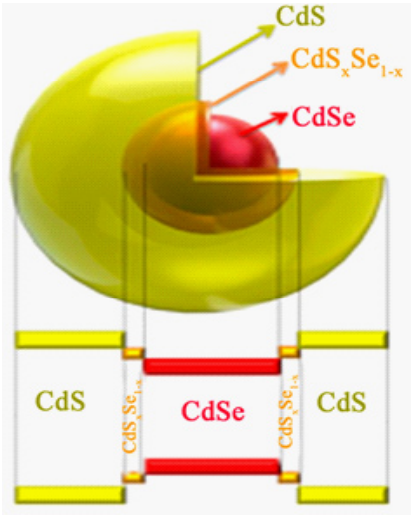

Figure 6: Representation of the process of inorganic passivation in QDs [31].

In a recent work, the passivation and functionalization processes have been performed in CdSe QDs, which have led to the formation of the CdS shell around the CdSe core of the QD as a function of the synthesis temperature [30] and the concentration of the stabilizer with external thiol grouping [31]. In addition, it was verified that the intensity of the luminescence is not proportional to the thickness of the shell. These methodologies were developed in order to increase the luminescence and biocompatibility of QDs, once cytotoxicity is related to the amount of $Cd2^{+}$ ions adsorbed on the surface of the CdSe QDs.

For biological and biomedical applications that use QDs as specific probes,the bioconjugation process is an important step. Depending on the specific chemical group in the surface of QDs, it is possible to attach a variety of biomolecules, including nucleic acids, proteins (e.g. avidin/streptavidin, albumin and antibodies), polysaccharides, and peptides. Therefore, the bioconjugation is performed after functionalization with the aim of coupling biological molecules to chemical bonds on QDs surface.

4. Biocompatibility of magic-sized quantum dots

Biocompatibility is the ability of nanomaterials in not causing toxicity or injury effects when in contact with live systems. Therefore, a biocompatible material must induce an appropriate host response [32,33]. The biocompatibility of materials is linked to their capacity of induce oxidative stress. An oxidative stress occurs when the production of oxidants surpass the antioxidant capacity in living cells. In this scenario, higher amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) will exceed basal levels. These sub-products of oxygen and nitrogen species are implicated in a number of pathological conditions, such as cardiovascular and neurodegenerative diseases [34]. The excess of ROS and RNS also cause different modifications on DNA and proteins, and enhance lipid peroxidation that, in turn, decrease the antioxidant activity of enzymes as catalase, superoxide dismutase and glutathione peroxidase [35].

The size and shape of materials have an important effect on the secretion of oxidants [36]. The size effect on oxidative stress became mostly evident when tested materialsare particles. Some publications demonstrated that immune cells, such as macrophages, are typically capable of phagocytizing particles below 5 μm [37,38]. The phagocytosis elicits ROS production as an attempt to eliminate the internalized material. However, the greatest cellular effects are observed with sub-micrometer particles. It is important to point out that nanocrystals can directly stimulate ROS formation or trigger their production through activation or inhibition of enzymatic pathways. In *vivo* and in *vitro* studies demonstrated that the toxicity of nanocrystals is closely associated with intracellular ROS production [39]. Regarding QDs, several noxious effects have been reported *byin vitro* assays. Cell death induced by necrosis and apoptosis associated with oxidative stress response, and many associated cellular mechanisms, such as elevated cytochrome c and calcium, upregulation of Fas, a death receptor on the surface of the cell that leads to apoptosis have been reported [40-42].

Metalsare one of the components of biocompatible materials that arecapable of inducing oxidative stress. In many medical implants, metallic materials undergo electrochemical corrosion, which releases products of degradation at the implanted sites, causing the formation or ROS and RNS [43].The main reason of oxidative stress in cellular systemscaused by QDs is the presence of metal ions. Cadmium ions adsorbed on the surface of the QDs interact with oxygen molecules in cells causing significant toxicity [44].However, the reduced number of ions adsorbed on QDs surfaces and the diameters of core and shell may improve antioxidant properties by altering the uptake of QDs by cells, consequently diminishing the harmful effects of oxidative stress [41,44]. Based on this, it is important to analyze the biocompatibility of QDs with different sizes by evaluating their potential to induce harmful effects and oxidative stress.

One of the reasons of QDs harmful effects is the cadmium ions released from the inorganic core [42]. The cytotoxicity of CdSe/CdS_xSe_{1-x} MSQDs functionalized with ahydroxyl group was investigated by fluorescence microscopy. Figure 7 shows the morphology of HeLa cells (A) incubated with CdSe MSQDs (B) and Etoposide (C). Acute cytotoxicity in HeLa cells was only observed after overnight exposure to Etoposide at 200 μM. HeLa cells incubated or not with CdSe MSQDs, observed by phase contrast microscopy, usuallyshow a thin and elongated form, which are homogeneously distributed throughout the culture and without cytotoxicity signs (Figure 5A and B). This specific result is due to the $CdSe_{1-x}S_x$ alloy at the shell protecting the CdSe core of the MSQDs, which prevents cadmium ion release,turning them more resistant to oxidation than other conventional QDs, from which reactions with oxygen usually cause $Cd2^+$ release from the core. On the other hand, the etoposide incubation led to various morphological abnormalities, as expected, such as reduced nuclear size, diverse degrees of chromatin condensation, cell shrinkage (round cells) and bleb formation on cell

surfaces, which indicate terminal apoptosis (Fig. 7C). Therefore, CdSe/CdS_xSe_{1-x} MSQDs did not alter the morphology of HeLa cells after 48-h incubation, suggesting no signs of cytotoxicity.

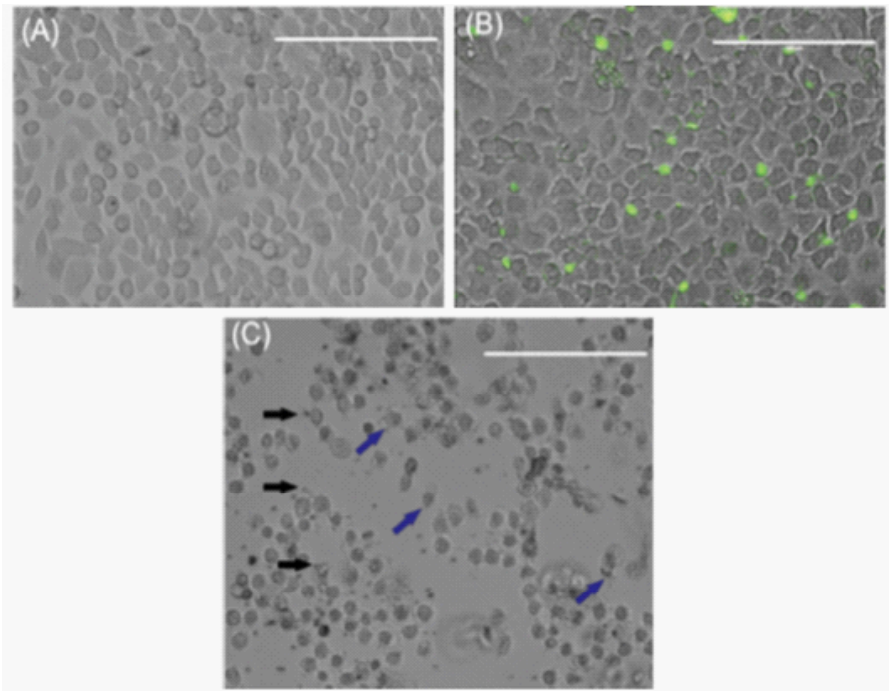

Figure 7: Morphological characterization of non-incubated (A) and incubated (B) HeLa cells submitted to MSQDs under phase contrast microscopy. Irregular shape and cellulardetachment (round cells) in etoposide treated cells are considered positive control of cytotoxicity (C). Black arrows indicate the formation of blebs on the cell surface as a result of the final death process. Blue arrows indicate the disruption of membrane cells. Scalebar = 200 μm [24].

In order to analyze what actually causes the cytotoxicity of cadmium chalcogenide MSQDs, we synthesized four samples with different concentrations of selenium during the synthesis, which directly affected the amount of Cd ions adsorbed on the surface of the MSQDs.

Figure 8a shows that levels of β-actin produced by cells were reduced after incubation as Se concentration increased [46]. Previously, it has been shown that the increased concentration of Cd ions adsorbed onto the MSQDs surface the higher the production of β-actin. The mRNA expression of themethallothionein gene(Figure 8b), an enzyme produced in the presence of metal ions, including cadmium [47,48], was lower when MSQDs presented less Cd ion concentration adsorbed at the MSQDs surface, suggesting that the cytotoxicity (Figure 8c) was due the concentration of Cd ions adsorbed on the surface of MSQDs; therefore, the more Se incorporated the smaller the harmful effects.

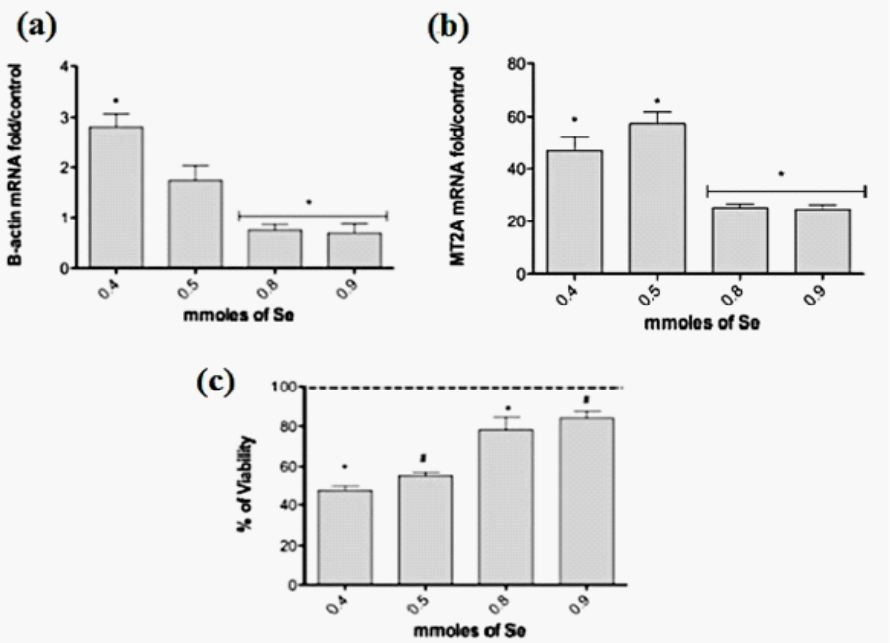

Figure 8. (a) MT2A expressions of HeLa cells exposed to CdSe MSQDs, synthesized at different Se concentrations, for 24 h. The mRNA expression was calculated as the ratio of fold to the control with POLR2A as an internal control. The results are expressed as means ±SE from two independent experiments. * represents $p < 0.05$ comparing 0.8 and 0.9 with other groups. Cytotoxicity and β-actin mRNA expression of CdSe MSQDs synthesized at different Se concentrations (0.4−0.9 mmol) exposed to HeLa cells for 24 h. (b) The mRNA expression was calculated as the fold of the expression related with the control with the POLR2A expression as the internal control. The results are expressed as means ± SE from two independent experiments. * represents $p < 0.05$ comparing 0.8 and 0.9 with 0.4 Se. (c) Cell viability was calculated as a percentage of the viable untreated cells (control). The viability of the control cells was considered 100% (dotted line). The results are expressed as means ± SE from two independent experiments. *# represents $p < 0.05$ comparing 0.4 with 0.8 Se and 0.5 with 0.9 Se, respectively [46].

Figure 9 shows a simplified model of the CdSe MSQDs synthesized at different Se concentrations. As Se concentration increases, Cd^{2+} density on the surface of the CdSe MSQDs decreases. This consequently decreases concentrations of surface defect levels (SDL) (pending Cd^{2+} bonds) and increases the intensity of excitonic emissions (Eexc). Not only does the density of Cd^{2+} surface level defects diminish, but the size of the MSQDs increases slightly at higher Se concentrations.

The representative scheme presented in Figure 10, corresponding to the behavior of the fluorescence spectra, justifies the biological results, in which cytotoxicity decreases with increasing Se concentration and, consequently, a decrease in the density of free Cd^{2+} on the surface of CdSe MSQDs. Also, the induction of metallothionein 2A (MT2A) expression in CdSe MSQDs, mainly at 0.4 and 0.5 Se concentrations compared to the 0.8 and 0.9 concentrations, may reflect the greater Cd^{2+} presence at the surface in the smaller Se concentrations. At the same time, MT2A acts as a ROS scavenger, protecting the cell and reducing the cytotoxicity. Based on this data, a possible reason for the cytotoxicity of our CdSe MSQDs is the disruption of the transcriptional and posttranscriptional pathways of different proteins, including β-actin.

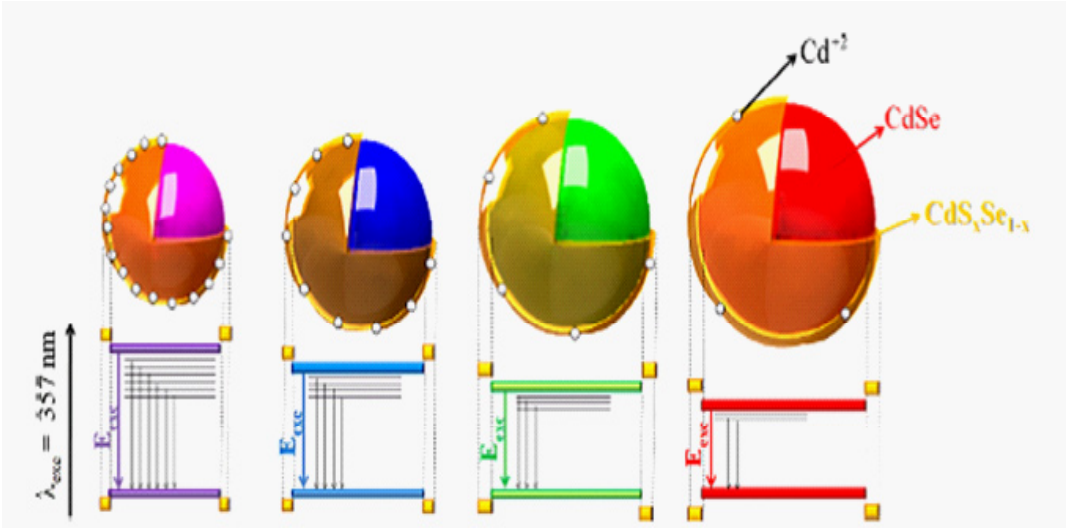

Figure 10: A simplified model of the CdSe MSQDs synthesized at different Se concentrations [46].

The cytotoxicity of CdSe/CdS_xSe_{1-x}/CdS MSQDs with external carboxylic chemical grouping was also investigated through MTT assays with the J774A.1 macrophage cell line (Figure 11A) and PBMCs from four healthy individuals under different concentrations of the MSQD (Figure 11B). The cell line incubated with different MSQDs concentrations did not show any significant cytotoxicity, except at concentrations ≥50 μg/mL during 24-h and 48-h incubation, in which the metabolic activity was significantly reduced. After 72-h incubation, a significant reduction of cell viability was also observed with MSQDs concentrations ≥10 μg/mL. PBMCs showed greater variations in cell viability without significant differences; however, with decreased viability as MSQDs concentration increased and during the time course of the stimulation assay.

In general, PBMCs showed greater viability than macrophages, and their variability was probably due to the heterogeneous cell population, which may be differentially affected by MSQDs. The cytotoxicity above 50 μg/mL may be justified by the aggregation of these QDs into organelles, such as mitochondria and lysosomes, altering their metabolic processes, and leading to a functional loss and death [44,49-51]. It is important to emphasize that such high concentrations of MSQDs are excessive for any cell line, and concentrations higher than 1 μg/mL are rarely used for specific applications.

The immunogenicity of MSQDs was analyzed by challenged PBMCs of healthy volunteers with 50 ng/mL MSQDs, the maximum concentration that did not show any cytotoxicity in PBMCs, as shown in Figure 12. There was no significant anti-inflammatory (IL-4, IL-10, TGF-β) (Figure 12, A) or proinflammatory (IL-2, IL-6, IL-8, TNF-alpha, IFN-gamma and IL-17A) (Figure 12, B) response, when compared to the negative control (untreated) in all incubation periods. TGF-β levels were relatively high in the immunogenicity assay, but cells treated with the MSQD were not significantly different from the control in none of the incubation periods, so we cannot infer that the CS-MSQD triggered TGF-β signaling.

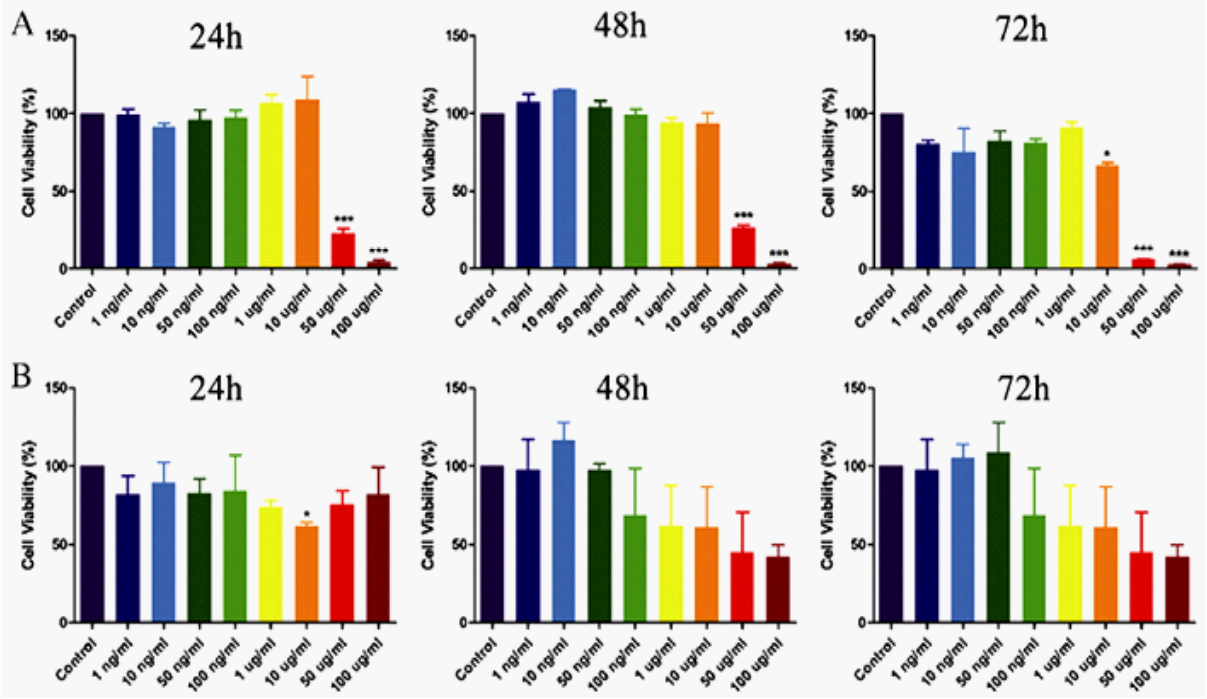

Figure 11: Cellular viability of the macrophage cell line J774A-1 (A) and PBMCs (B) from healthy individuals submitted to the MTT colorimetric assay after incubation with CdSe/CdS_xSe_{1-x}/CdS MSQDs. The MSQDs concentration ranged from 1 ng/mL to 100 µg/mL monitored from 24 to 120 h. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The results are expressed as means ± SD (standard deviation) from three independent experiments. *Statistical significance was considered when $P < 0.05$ in relation to control cultures (one-way ANOVA with post hoc Bonferroni's test) [52].

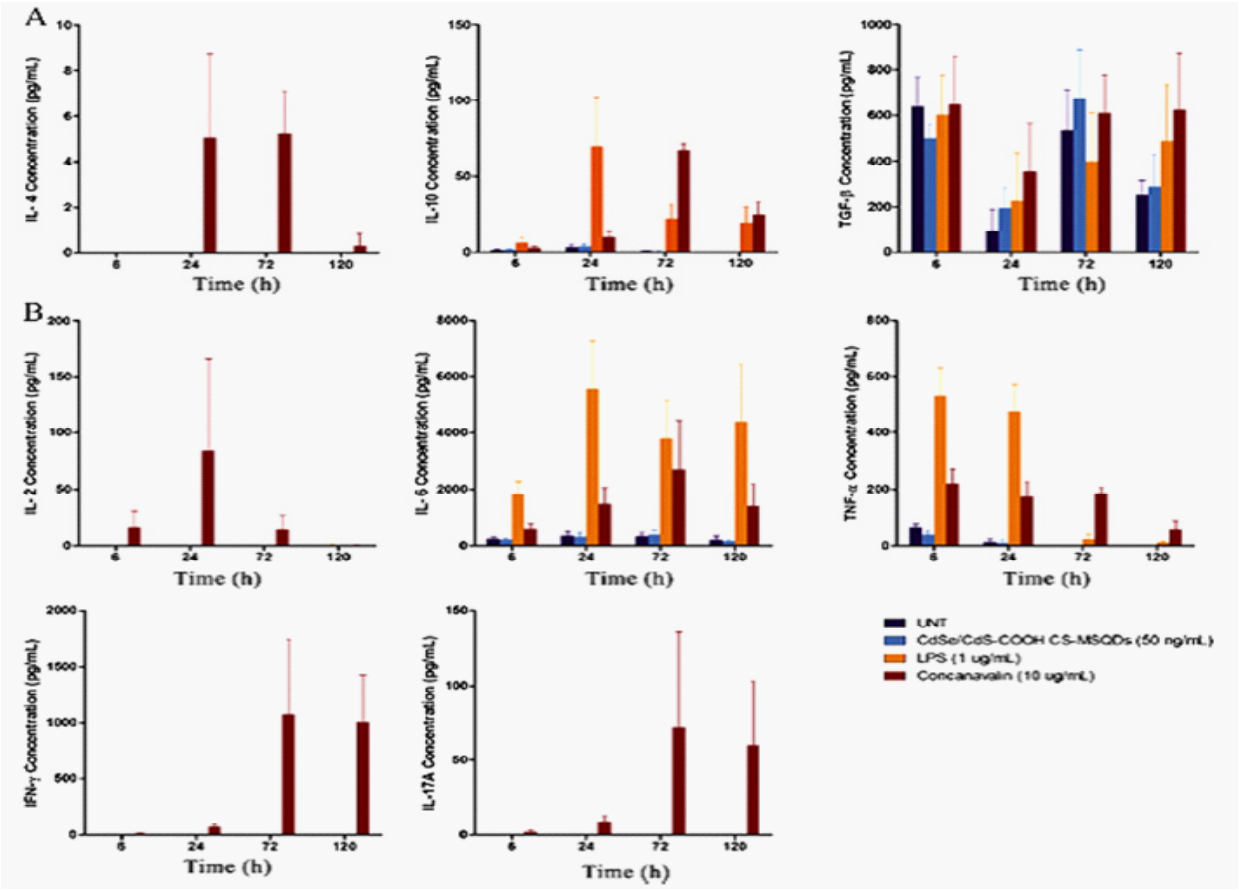

Figure 12: Th1/Th2/Th17 cytokines panel determination using cytometric bead array (CBA) assay of human PBMCs incubated with the CdSe/CdS_xSe_{1-x}/CdS MSQDs. Human PBMCs (N = 3) were treated with 50 ng/mL of MSQD for 6, 24, 72 and 120 h. Supernatants were harvested and assayed to determine the concentration of 8 different cytokines. Response profiles of pro- and anti-inflammatory cytokines were established. There were no statistical differences between QD stimulus and controls (untreated; UNT) (two-way ANOVA with post hoc Bonferroni's test). The lipopolysaccharide (1 μg/mL) and concanavalin (10 μg/mL) were used as positive controls of the reaction [52].

It is important to investigate the TGF-β signaling pathway due to its critical role in many biological processes, which is defined by the cell type and the micro environment [53,54], but while in infections it protects against excessive inflammation, it may also promote immune evasion and chronic infections. In autoimmune diseases, it leads to the loss of tolerance to self-antigens, and in cancer it acts as a potent inhibitor of cell proliferation and as tumor suppressor during tumorigenesis [55].

These pleiotropic and opposing roles of TGF-β may raise questions about the CS-MSQD applications in vivo either for diagnostics or drug delivery, especially for tumor imaging. However, in order to characterize the inflammatory or anti-inflammatory profiles of the immune response, one should consider common cytokines signatures and not a cytokine alone. In the present study, none of the evaluated cytokines showed a significant increase in the presence of the MSQD. So, the lack of immunogenicity corroborates the biocompatible nature of the MSQD. This is probably due to the ultra-small size, which may not be recognized by receptors, entering the cells by passive diffusion.

In summary, besides the toxicity analyses of new QDs, it is important to test their immunogenicity in order to prevent undesirable or unexpected response for *in vivo* applications.

5. Genotoxicity analyses of ZnO and Tio_2 nanocrystals

DNA damage can occur spontaneously through normal biological processes or asa result of direct or indirect interaction of DNA with chemical, physical or biological agents. The rapid development of nanotechnology allowed the increasing human environmental exposure to engineered nanocrystals in the form of industrial products such as dyes, paints, clothing, electronics, sunscreens, cosmetics, personal care products, food packaging, drug delivery systems, therapeutics, biosensors and others, generatingthe interest of researchers to investigate the potential hazards of NCs to DNA.

At present the genotoxic evaluation of NCs have been performed by means of different traditional genotoxicity tests, such as bacterial reverse mutation assay (Ames assay), *in vivo* and *in vitro* chromosomal aberration tests, in vivo comet assay, micronucleus test *in vivo* and *in vitro,* unscheduled DNA synthesis assay, somatic mutation and recombination test (SMART), among others [56-61].

In vivo studies offer many advantages, such as the study of the bioavailability of NCs to sensitive target cells and can increase the knowledge on the possible genotoxic potential risk associated with NCs exposure [62]. Nevertheless, the commonly used *in vivo* mammalian tests appear to be ill adapted to tackle the large compound sets involved, due to throughput, cost, and ethical issues. Non-mammalian animals, such as the common fruit fly Drosophila melanogaster are good candidates for the development of high-throughput genotoxicity tests due to their quick reproductive cycles, greater ethical acceptance, and smaller infrastructure needs [63], as it has a mammalian-like enzymatic system that allows the breakdown of xenobiotic agents. Many basic biological, physiological, and neurological properties are conserved between mammals and D. melanogaster, and nearly 75% of human disease-causing genes are believed to have a functional homolog in the fly [64]. In addition to presenting highly reliable and reproducible results, it allows to quantify the recombinogenic activity against the mutagenic activity of different compounds and mixtures [65]. Recently, a review article on Drosophotoxicology Chifiriuc et al. [66] has been published as an emerging research area for assessing nanocrystals interaction with living organisms. Additionally,it has also been presented the suitability of D. melanogaster as an in vivo model to determine potential side effects of nanomaterials [67].

On the other hand, the toxicological relevance of in vitro assays as the micronucleus(MN) test is due to the fact that it is a multi-target genotoxic endpoint that assesses not only theclastogenic and aneugenic events, but also some epigenetic effects. It is simple to score, accurate, applicable to different cell types, predictive for cancer, amenable for automation, and easily measured in experimental both *in vitro* and *in vivo* systems [68]. MN is a small nuclei-like structure enveloped by a nuclear membrane, located in the cytoplasm outside of the main daughter nuclei, which represent either a whole lagging chromosome (resulting of an aneugenic event leading to chromosome loss) or an acentric fragment of a chromosome (resulting of clastogenic event) that was retained in anaphase and not incorporated into one of the daughter nuclei during cell division [69,70]. The *in vitro* Cytokinesis Block Micronucleus (CBMN), established inhuman peripheral blood lymphocytes has several advantages, such as the speed and easiness, no requirement for metaphase cells, and reliable identification of cells that have completed only one nuclear division.

For these reasons, we highlight the Drosophila wing somatic mutation and recombination test (SMART), also known as the wing spot test, and the CBMN assay as potential methodologies to assess the genotoxic potential of NCs.

5.1 Somatic mutation and recombination test in D. melanogaster

The SMART provides a rapid means to assess the potential of a chemical to induce loss of heterozygosity (LOH) resulting from gene mutation, chromosomal rearrangement, chromo

some breakage, or chromosome loss. This bioassay makes use of the wing-cell recessive markers *multiple wing hairs (mwh, 3–0.3)* and *flare (flr3, 3–38.8),* located at the tip and roughly in the middle of the left arm of chromosome 3, respectively. The LOH leads to uncovering and expression of the recessive marker gene(s) in the larval imaginal disk cells. They give rise to clones of mutant cells showing up as mosaic spots on the wings [71,72]. Two crosses are usually carried out to produce the experimental larval progeny: [1] ST cross, flr^3/In(3LR)TM3, ri p^psep l(3)89Aa bx^{34e} e Bd^S females crossed with mwh males [73,74];[2] HB cross, ORR; flr^3/ In(3LR)TM3, ri p^psep l(3)89Aa $bx3^{4e}$ e Bd^S females crossed with *mwh* males [75]. The latter cross is characterized by improved sensitivity to a number of promutagens and procarcinogens owing to high levels of constitutively expressed cytochromes P450. Both crosses produce two types of progeny: (i) marker-heterozygous (MH) flies; (ii) balancer-heterozygous (BH) flies, as shown in Figure 13.

In MH individuals, single spots (*mwh or flr)* can be produced either by point mutation, certain types of chromosome breakage event (deletion) or by mitotic recombination. However, we cannot tell how much each mechanism contributes to the total of spots recovered. On the other hand, the presence of twin spots proves that mitotic recombination is induced, as these spots result from mitotic crossing-over between the *flr* locus and the centromere. Figure 14presents a twin spot found in the blade of *Drosophila* wing showing normal and mutants flare and multiple wing hairs.

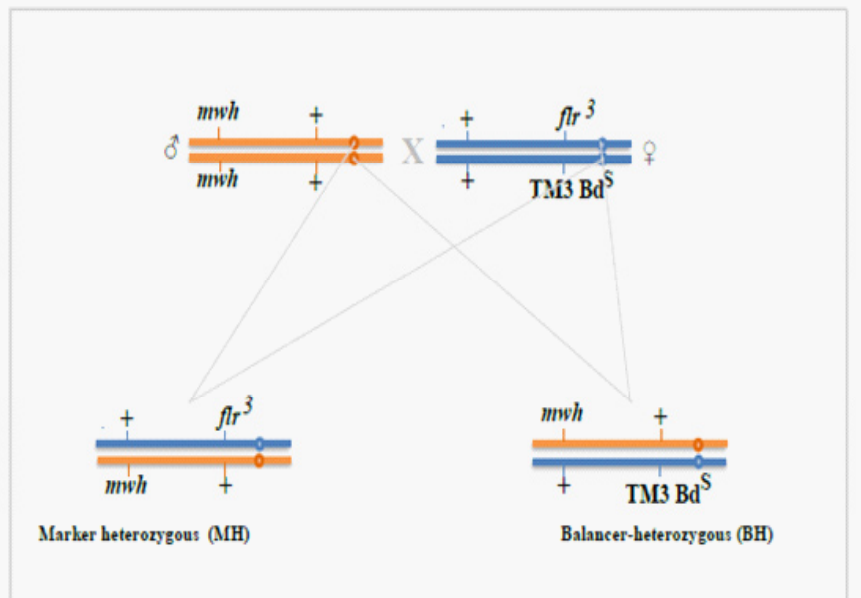

Figure 13: Standard (ST) cross: mwhmales crossed with flr3/In(3LR)TM3, ri pp sep l(3)89Aa bx34e e BdS- females producing two types of progeny: marker-heterozygous(MH) flies and balancer-heterozygous (BH) flies.

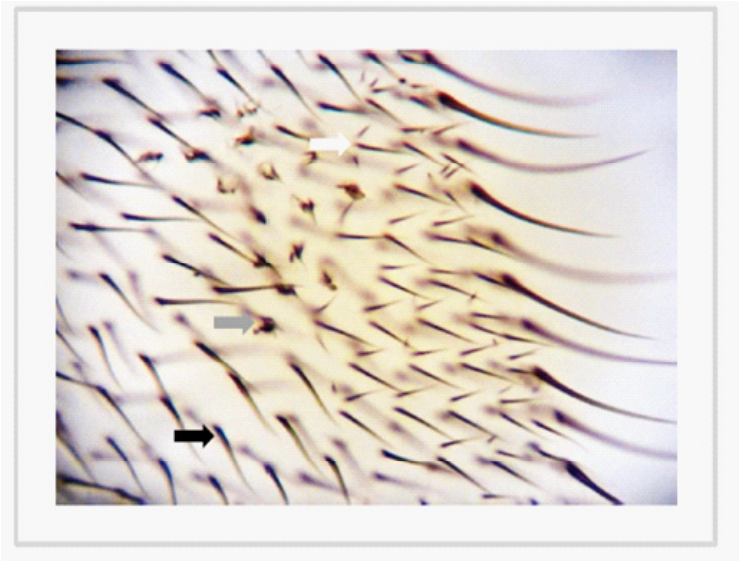

Figure 14: Twin spot found in the blade of Drosophila wing showing normal hair (black arrow), flare hair (gray arrow), and multiple wing hairs (white arrow).

In BH individuals, the flr marker is absent. The spots that can be recovered are therefore mwh single spots. All recombination events are suppressed or eliminated owing to inversion heterozygosity brought about by the presence of the multiply inverted balancer chromosome. As a rule, therefore, the frequencies of mwh clones observed on the wings of BH flies are always lower than those observed on the wings of MH flies [73,76]. Many hundreds of chemicals have already been tested using SMART, including NCs [60,62,72,77-79].

The wing SMART in *D. melanogaster* has been widely used to assess genotoxic potential of different chemicals compounds, including NCs. Here we only present some selected results obtained with ZnO NCs and TiO_2 NCs.

The mutagenic and recombinogenic potential of an amorphous and ZnONCs (20 nm) using both crosses of the wing SMART of D. *melanogaster* has been evaluated [60]. Third instar larvae were fed chronically (approximately 48 h) with four different concentrations (ranging from 1.56 to 12.50 mM). In the ST cross, the amorphous and ZnO NCs (20 nm) were not mutagenic. Nevertheless, MH individuals from the HB cross that were treated with amorphous (6.25 mM) and ZnO NCs (12.50 mM) displayed a significant increased number of mutant spots when compared with the negative control. The increase in mutant spots observed in *D. melanogaster* was generated due to mitotic recombination, rather than mutational events. Our results suggest that ZnO mutagenicity can be related to particle size and shape. As ZnO NCs had a uniform feature with an average size of 20 nm, it could enter the cell homogeneously and cause mutagenicity at higher concentrations. On the other hand, as the amorphous ZnO comprises a mixture of structures of different shapes and sizes, its mutagenicity could be variable, according to the size and shape of the crystals and its ability to enter the cell. Consequently, mutagenic effects related to the dose could not be expected.

In agreement with the previous results, it has also been described a lack of genotoxic ac

tivity of different concentrations (6, 12, 18 or 24 mM) of ZnO NCs and ZnO bulk assayed with the ST cross of the *Drosophila* wing-spot test [80]. Nevertheless, when these authors tested both particles in haemolymph cells of *Drosophila* by the comet assay, a significant increase in DNA damage was observed at the higher dose applied of ZnO NCs. The authors concluded that the genotoxic potential of ZnO NCs could be considered low or weak because it promotes only primary genetic damage restricted to high-dose exposures, which can be easily repaired.

On the other hand, ZnO NCs with an average size of 50 nm, assayed with the ST cross showed a significant increase in the frequency of mutant clones in the flies exposed to the concentration of 1.2 mg/mL [81].

The genotoxic effects of 3.4 and 6.2 nm TiO_2 anatase NCs (A3.4 and A6.2) and 78.0 nm predominantly TiO_2 rutile NCs (R78.0) through the *Drosophila* wing SMART were also evaluated [79]. Third instar larvae obtained from standard (ST) or high bioactivation (HB) crosses were treated with a solution containing TiO_2 (A3.4, A6.2 or R78.0) NCs at concentrations ranging from 1.5625 to 100.0 mM. In the ST cross, no mutagenic effects were observed. However, in the HB cross, TiO_2A3.4 NCs were mutagenic at 1.5625 and 3.125 mM, while theR78.0 NCs increased mutant spots at all concentrations tested except 3.125 mM. Only the smallest anatase TiO_2 NCs induced mutagenic effects. Therefore, both anatase and rutile TiO_2 NCs induced mutagenicity.

Negative results in the ST cross had already been observed previouslywith TiO_2 NCs (2.3 nm) at concentration ranging from 0.1 to 10 mM [78] and with TiO_2 anatase NCs (<25 nm particle size, surface area 45–50 m2/g, 99.7% purity) as well as their TiO_2anatase bulk form (TiO_2 bulk, 45 μm, 99% purity) [77]. Third instar larvae were fed with both compounds at different concentrations (0.08, 0.40, 0.80 and 1.60 mg/mL, respectively). None of the concentrations of TiO_2 NCs and TiO_2 bulk induced significant increases in the frequency of mutant spots. Nevertheless, in this study, the authors evaluated the same concentrations of both nano- and bulk forms of TiO_2 with the comet assay in Drosophilahemocytes. TiO_2 NCs at concentrations ranging from 0.40 to 1.60 mg/mL induced a significant increase in DNA damage, with a direct dose-response pattern.

5.2 Cytokinesis-block micronucleus (CBMN) assay

The *in vitro* Cytokinesis Block Micronucleus (CBMN) Assay is one of the standard cytogenetic tools used widely as a screening *in vitro* test for structural or numerical chromosomal anomalies induced by clastogenic or aneugenic agents (‘‘spindle poisoning’’) [82]. This technique consists in adding to cell cultures cytochalasin-B, an inhibitor of the mitotic spindle that prevents cytokinesis, producing binucleated cells, allowing a more accurate MN score and excluding the dividing cells from the non-dividing cells to enhance the reliability by reducing the incidence of false positive data [83].

Figure 15 shows a binucleated cell with a MN. The CBMN assay has been used to evaluate clastogenic and aneugenic effects of different compounds, including NCs [60,78,79,84].

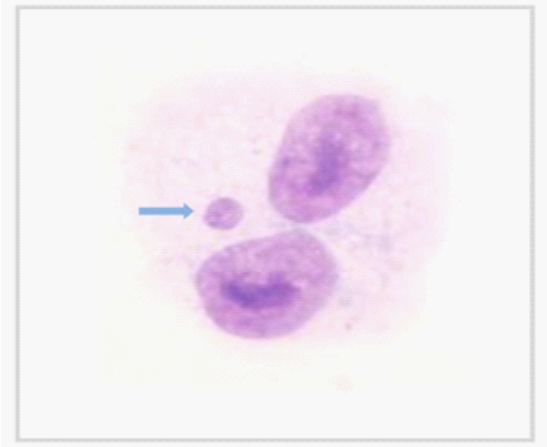

Figure 15: Optical microphotography of V79 binucleated cells with micronucleus (arrow) (Giemsa; total increase: 1000×).

Here, we will only present selected results obtained with ZnO NCs and TiO_2 NCs using an *in vitro* CBMN Assay. The genotoxic potential of ZnO NCs of 20 nm using the Chinese hamster lung fibroblast V79 cells has been evaluated [60], and results demonstrated that were not dose-dependent and indicated that only higher concentrations of ZnO NCs were toxic and able to induce genotoxicity in V79 cells.

In agreement with the previous data, significant increase of MN was observed in human epithelial HEp-2 cell line treated with ZnO NCs at high concentrations (50 and 100 µg/ml) (Osman et al., 2010); in A549 human lung carcinoma cells treated with 50 µg/ml ZnONC [85]; in human peripheral blood mononuclear cells treated with ZnO NCs at a concentration of 45 µg/ml [86];and human peripheral lymphocytes treated with ZnO (diameter of 45 nm and fragment size of 450 nm) at 10 and 15µg/ml [88].

The genotoxic effects of 3.4 and 6.2 nm anatase TiO_2 NCs and 78.0 nm predominantly rutile TiO_2 NCs were also evaluated through CBMN assay using V79 cells treated with 30, 60 or 120 mM anatase 3.4 or A6.2 TiO_2, and 976.5, 1953.0 or 3906.0 mM rutile 78.0 TiO_2 [79]. Only the smallest anatase (3.4 nm) TiO_2 NCs significantly increased the frequency of MN at the highest concentration (120 mM), showing a genotoxic effect. No significant differences in MN induction were observed in the groups treated with 30 and 60 mM A3.4 TiO_2 when compared to negative control ($p>0.05$). The results also demonstrated lack of genotoxic (clastogenic/aneugenic) effects for A6.2 and R78.0 TiO_2 at all concentrations tested.

Similar results were observed else where [89], in which the genotoxicity of three reference TiO_2 nanomaterials (NM) was also investigated in human bronchial epithelial BEAS 2B cells. The BEAS 2B cells were submitted to NM100 (anatase, 50–150 nm), NM101 (anatase, 5–8 nm) and NM103 (rutile, 20–28 nm) TiO_2 dispersions at 1, 5 or 15 µg/ml (0.2–3.2 µg/

cm2) for 48h, and increase MN was observed only by the rutile NM103 in the lowest concentrations tested (1 and 5 μg/ml).

We propose the *Drosophila* wing SMART and the CBMN assays as *in vivo* and *in vitro* systems, respectively, to screen NCs for potential genotoxicity, mutagenicity and recombinogenicity. Nevertheless, due to the contradictory results found in the literature, further research is necessary to clarify the NCs action mechanisms, highlighting the importance of using more than one genetic end-point.

6. Biocompatible luminescent probes using magic-sized quantum dots

In a recent work, the CdSe/CdS_xSe_{1-x}/CdS-COOH MSQDs has been successfully conjugated to a specific antibody for breast cancer aiming their use as biological probe, as shown Figure 16. Additionally, it has been shown that this MSQDs presented low cytotoxicity and no immune response, enabling their use for *in vivo* applications.

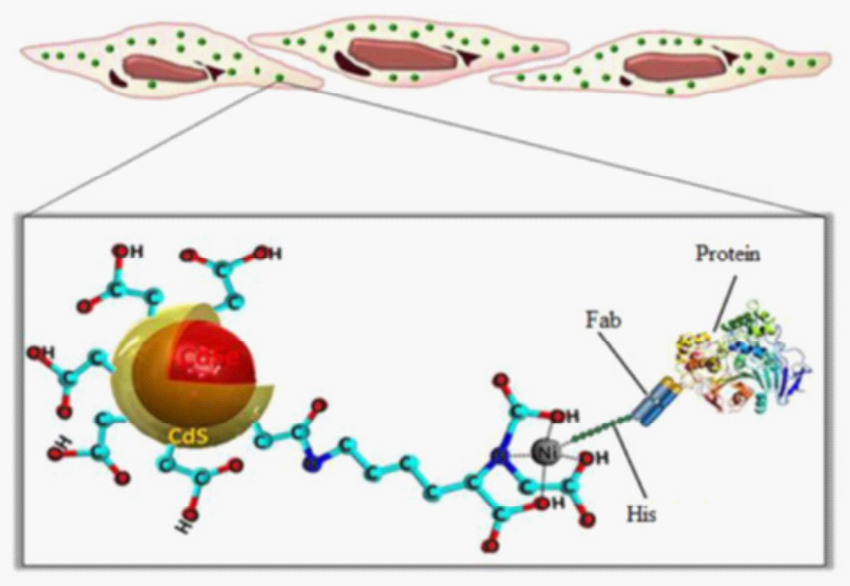

Figure 16: Representation of bioconjugation process performed [52].

The bioconjugation procedure of the MSQD-COOH with an Fab antibody by means of the compound containing nickel ion (precursor nickel chloride $NiCl_2$) together with nitriloacetic acid (NTA • Ni^{2+}) is described in Figure 17. Thus, the development of CdSe/CdS_xSe_{1-x}/CdS MSQDs complex with NTA•Ni^{2+} moieties exposed at the surface, which are capable of specifically recognizing histidine-tagged Fab antibodies, was used to detect breast cancer cells by immuno fluorescence.

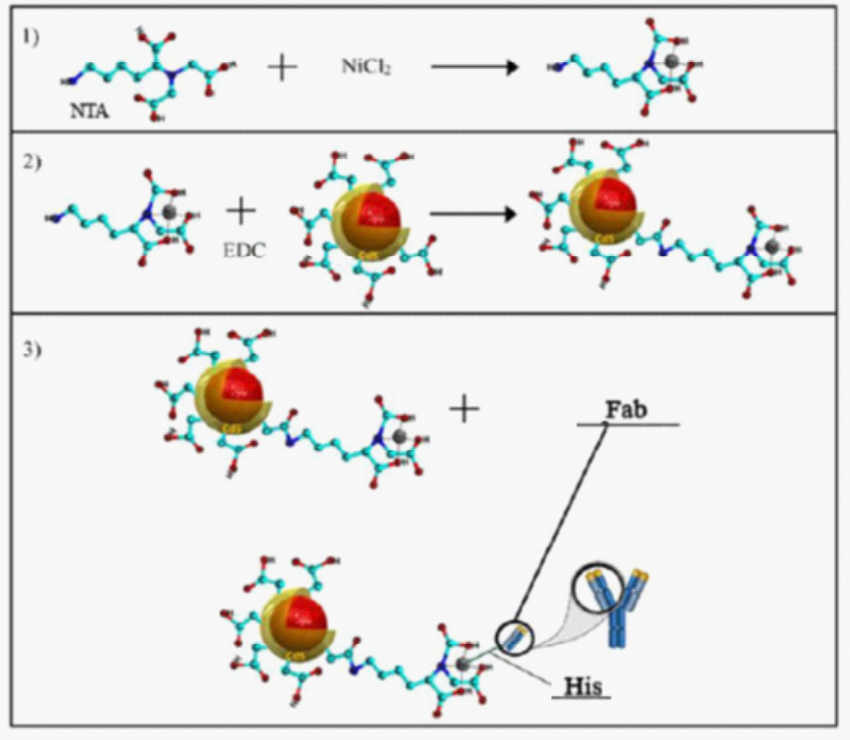

Figure 17: Schematic representation of the reactions to bioconjugateCdSe/CdS_xSe_{1-x}/CdSMSQDs to an antibody specific for breast cancer. MSQDs were functionalized with the NTA • Ni^{2+} complex, which interacted with the histidine tail of the Fab antibody.

In order to confirm the functionalization of the MSQDs-COOH with an NTA • Ni^{2+} complex, and the bioconjugation with the Fab antibody, the Fourier Transform Infrared (FT-IR) spectroscopy was used, as shown in Figure 18.

The spectrum of MSQDs observed at the broad band 3423–3292 cm−1 shows the specific characteristics of OH group and adsorbing water(Figure 18). The presence of bands at 1280, 1556, 1357 cm^{-1} and 1713–1602 cm^{-1} are associated with vibrations of hydroxyl, antisymmetric νC=O, asymmetric νCOO− and symmetric νCOO⁻ groups, respectively [90]. These peaks confirmed that MSQDs are functionalized with carboxyl group at the surface. Moreover, in the spectrum of MSQDs also showed that the peak at 2600 cm^{-1} of stretching modes of S–H bonding disappeared, confirming the covalent bond formation between S of the stabilizer molecule and cadmium ions (Cd^{2+}) of the MSQDs, forming a shell of CdS [31]. Furthermore, the spectra of the MSQDs showed bands around 609 cm^{-1} and 1622 $cm^{-}1$ that reinforce the formation of the CdS shell around the CdSe MSQDs [31]. The shell prevents Cd^{2+} release from the CdSe core and makes the MSQDs less cytotoxic, which is confirmed by tests with specific cellular metabolic activity under stimuli of MSQD.

The FT-IR spectrum of the MSQDs coupled with antibody presented pronounced bands of amide I and II vibrational stretches at 1645 and 1555 cm^{-1} (squared box), respectively, and shifts in the N–H stretching region (3500-3000 cm^{-1}) [91]. This demonstrates that the MSQDs are covalently attached to NTA • Ni^{2+} moieties through an organic linkage and not through nonspecific binding interaction, and coupling of CS-MSQD with Fab antibody was carried out

through the interaction of Ni^{2+} with the histidine tag present in the antibody [92], coordinated by imidazole binding, confirmingbthe conjugation as demonstrated in Figure 17 [93]. It was also noted that the vibrational modes corresponding to Fab antibody (region from 550-1550 cm^{-1}) remained intact, demonstrating that coupling did not affect the 3D structure of the conjugated Fab antibody, and did not alter the specificity, the cytosolic conformational epitope of cytokeratin 10 (CK-10) [94].

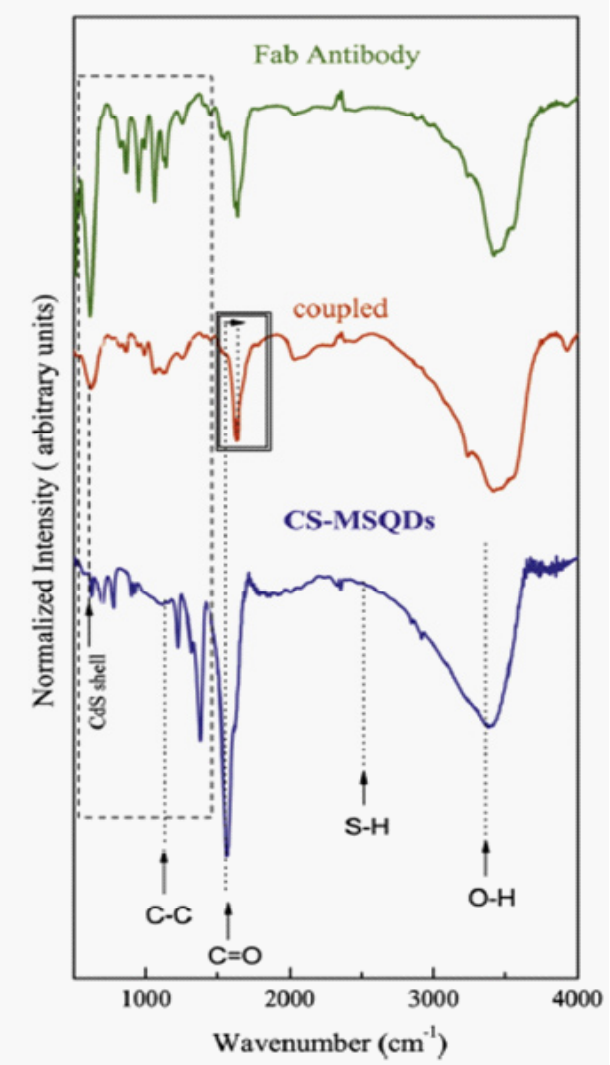

Figure 18: FT-IR spectra Fab antibody and of the CdSe/CdS_xSe_{1-x}/CdSMSQDs pre- and post-bioconjugation [52].

It is important to check the bioconjugation also by means of biological assays. Thus, imaging analysis were performed to test the efficiency of MSQDs coupled to a specific breast cancer Fab antibody in an immuno cytochemistry assay. Figure 19 shows a highfluorescence intensity demonstrating that the MSQDs were efficiently conjugated to Fab antibodies with high specificity to breast cancer cells when compared with controls (non-labeled MSQDs). The cell auto-fluorescence in the control (Figure 15A) is similar to that observed in Figure 15B, which was expected because MSQDs in Figure 15B were washed off, and only few random spots could be seen. The higher magnification setting in Figure 15A was used to demonstrate the auto-fluorescence behavior of cells without MSQDs. However, the most striking difference is shown in Figure 15C, where specific immunoagglutination has occurred.

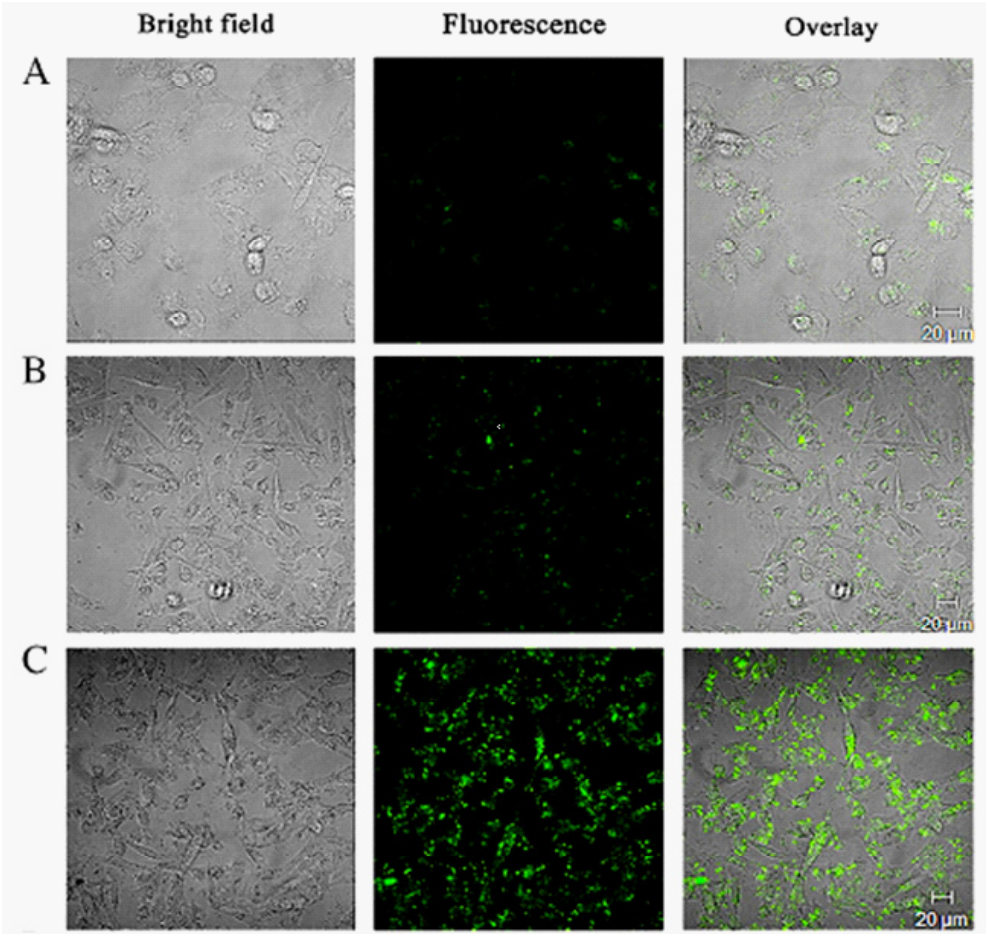

Figure 19: Specific cytosolic labeling of the breast cancer (BC) cell line MDA-MB-231 with CdSe/CdS_xSe_{1-x}/CdS MSQDs conjugated with a specific BC Fab antibody analyzed by confocal microscopy. Cells were incubated with MSQDs for 1 h. (A) MDA-MB cells (control); (B) MDA-MB cells incubated with MSQDs; (C) MSQDs conjugated to Fab antibody. Images obtained in Zeiss LSM 510 Meta Confocal Microscope with laser emission 530 nm (green channel) [52].

The CS-MSQD presents high fluorescence stability, low toxicity, no immunogenicity, and biocompatibility, demonstrating to be a powerful tool for diagnostic and imaging techniques. These highly desirable characteristics may also present great potential for drug delivery, which must still be demonstrated.

7. References

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Chapter 3

Inborn Errors of Metabolism

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1. Introduction

Inborn errors of metabolism (IEM) are a group of inherited metabolic disorders leading to enzymatic defects in the human metabolism. As its name implies, inborn errors means birth defects in newborn infants which passed down from family and affecting metabolism. Hence, it is called Inborn errors of metabolism or inherited metabolic disorders. IEM can appear at birth or later in life such as phenylketonuria, albinism, lactose intolerance, gaucher disease, fabry disease etc. IEM refers a condition where in body's metabolism is affected due to genetic disorders. The cause of IEM is mutations in a gene that code for an enzyme leading to synthesis of defective enzyme activity or deficiency of an enzyme that affects the normal function of a metabolic pathway. The main indication of IEM is an excess storage or accumulation of specific metabolites in tissues, organs and blood which further manifest to health diseases. In last decades, several hundreds of different IEM have been identified. Most IEM are rare but some are life threatening. Although, most people do not know what inherited metabolic disorders are and may never have heard of them.

Therefore, in this chapter, you are going to study the basic concept, genetic basis and metabolic consequences of inborn errors of metabolism. You will learn about metabolic defective enzymes, clinical symptoms, diagnosis, and treatment of metabolic disorders of amino acids, carbohydrates, lipids, purines and pyrimidines.

Before learning to inborn errors of metabolism, it will be helpful for you to recall the basic knowledge of amino acids, carbohydrates, lipids, purines and pyrimidines, and also study about the metabolic pathways, enzymes, Mendelian Laws of Genetics from your Biochemistry and genetics textbooks.

2. Inborn errors of metabolism: Basic concept and genetic basis

The British physician, Archibald Garrod (1857-1936) coined the term inborn errors of metabolism in 1902 who discovered the first metabolic disorder "Alkaptonuria" in humans. Later, he described other metabolic disorders: albinism, cystinuria, porphyria, pentosuria. He was the first man who defined the biochemical basis of Alkaptonuria [1]. His pioneering work led to the foundation for the development of a new field of Human Biochemical Genetics. However, Garrod's work was not well accepted in early nineteenth century because the nature of gene was not fully understood [2].

Later, George Beadle and Edward Tatum scientifically proved his findings in 1941 and proposed the one gene–one enzyme theory. This theory proposed that each gene has a genetic information to synthesize a protein (enzyme). However, this theory is generally (but not exactly) correct.

2.1 Genetic basis

Mutation in single genes is the genetic basis of inborn errors of metabolism. A mutation is the change of human genome (genes) which can arised by exposer of viruses, pathogenic bacteria, UV-radiations, unhealthy foods and environmental factors. Thus, mutations causes abnormalities in genes (genetic disorders) which consequently resulting in inherited human diseases. As per Mendelian Laws of Inheritance, all genotypic information are inherited from one generation to another which determine phenotypic and genotypic characteristics in human beings [3]. Most IEM are genetically-transmitted diseases result from alteration in either autosomal recessive (non-sex chromosomes) or X-linked recessive (sex-linked chromosomes). Genes disorders inherit as either dominant or recessive mode in human beings [4].

Let us understand inheritance pattern. Fig.1.1 shows the autosomal recessive inheritance pattern of a family in which father and mother have normal two carrier genes which transfer to children. Individuals with one working copy and one non-working copy of the gene are called carriers. Recessive genetic disorders determined by two carrier genes, one inherits from the father and one from the mother. Therefore, the chance to have carrier genes in autosomal inheritance pattern is as follows:

1. Child (A) will be normal with 25% chance of inheritance

2. Child (B and C) will be 50% carrier like the parents but usually will not appear symptoms and

3. Child (D) will have 25% chance to get an inherited disorder when an individual receives the same abnormal gene for the same trait from each parent.

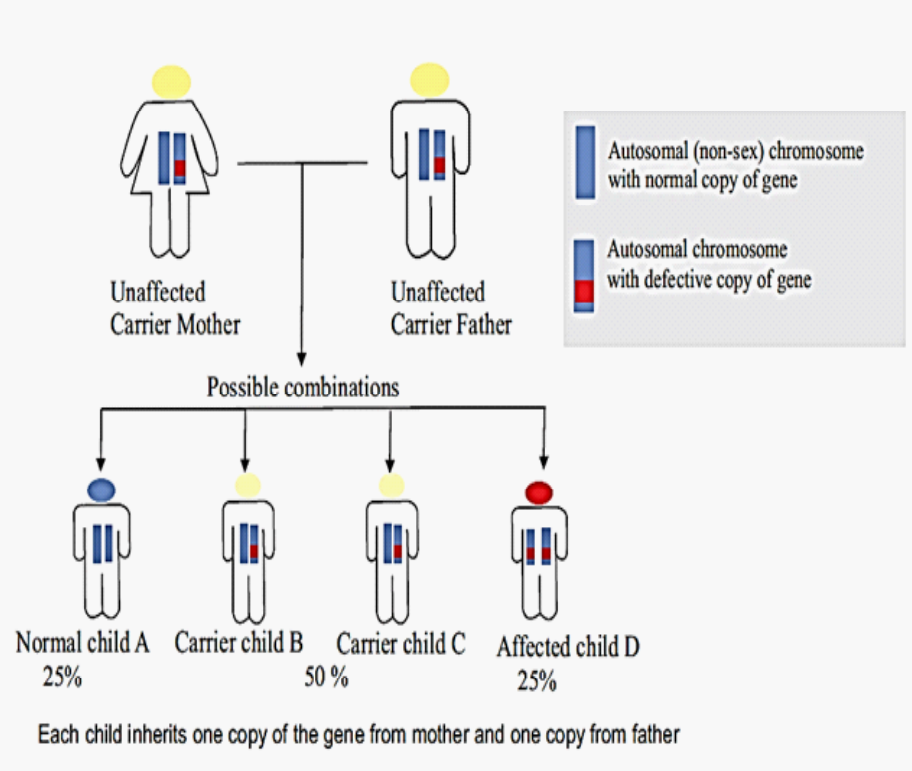

Figure 1.1: Autosomal recessive Inheritance:
Source: http://learn.genetics.utah.edu/content/disorders/singlegene/

3. IEM: Metabolic consequences

Let us understand how gene mutation affecting a metabolic pathway at biochemical level. As you know metabolism is basically energy production and consumption process that undergoes in a series of chemical reactions occurring within a cell. Metabolic pathways are required for maintenance of the human body in healthy state. All metabolic events are driven by enzymes that are catalytic proteins and their main function is to accelerate the rate of biochemical reactions, without being altered during that process. Enzymes play a crucial role in the metabolism of food stuffs, such as proteins, fats, and carbohydrates. If an error in the normal metabolic process due to deficiency of an enzyme caused metabolic abnormalities.

Fig.1.2 revealed the biochemical basis of inherited metabolic defect in which a gene code for an enzyme that catalyzes a specific substrate into products in a biochemical pathway. On the other hand, the gene is mutated that codes an enzyme leads to the formation of a defective enzyme. As a result, a metabolic blockage occurs in a specific metabolic pathway resulting in elevation of substrate concentration and product shortage in cells [5].

Therefore, the possible metabolic consequences of IEM are:

- Accumulation of a substrate,
- Accumulation of intermediate Metabolites,
- Lack of an essential product,
- Interfere with normal metabolic function.

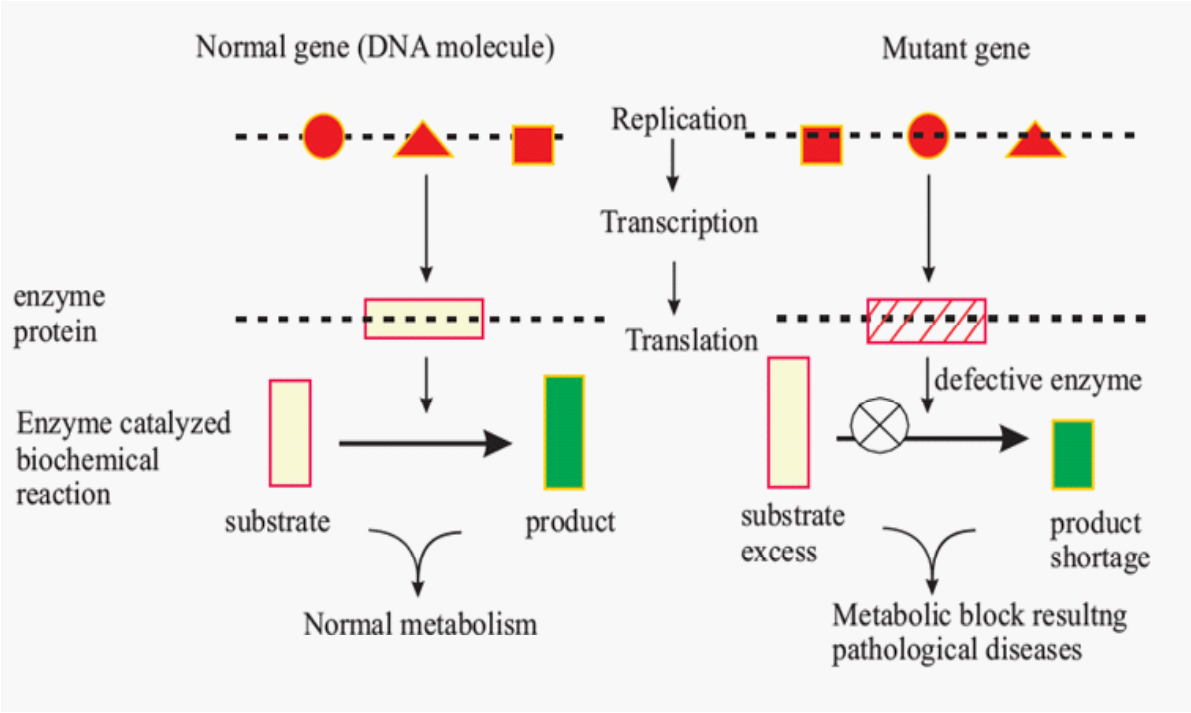

Figure 1.2: Inborn errors of metabolism
Source: https://www.britannica.com/science/metabolic-disease

You have studied till now genetic basis and metabolic consequences of the Inborn errors of metabolism. The disorders of IEM are classified on the basis of defect in specific enzymes involved in the metabolism of amino acids, carbohydrates, lipids, purines and pyrimidines. Let us discuss one by one.

Note: Defective enzymes are shown by ⊗ red summing junction in all metabolic reactions

4. Disorders of amino acid metabolism

As you know, amino acids are the building block of proteins. Amino acid metabolic disorders are defined by accumulation of metabolic intermediates that cause specific tissue and organ damage [6]. The amino acid-metabolic disorders along with other characteristics are listed in Table 1. The chemical reactions along with enzymes involved in the catabolism of phenylalanine and tyrosine are shown in Fig.1.3. Let us discuss the disorders of amino acid metabolism.

4.1 Phenylketonuria (PKU)

Phenylketonuria is a common autosomal recessive inborn error of phenylalanine leads to intellectual disability if untreated. The estimated incidence of PKU 1 in 10,000 newborns. Phenylalanine is an essential amino acid which means it cannot synthesize in the body and must be taken from the diet. In PKU, the first step of phenylalanine catabolism is affected due to the defective catalytic activity of phenylalanine hydroxylase enzyme.

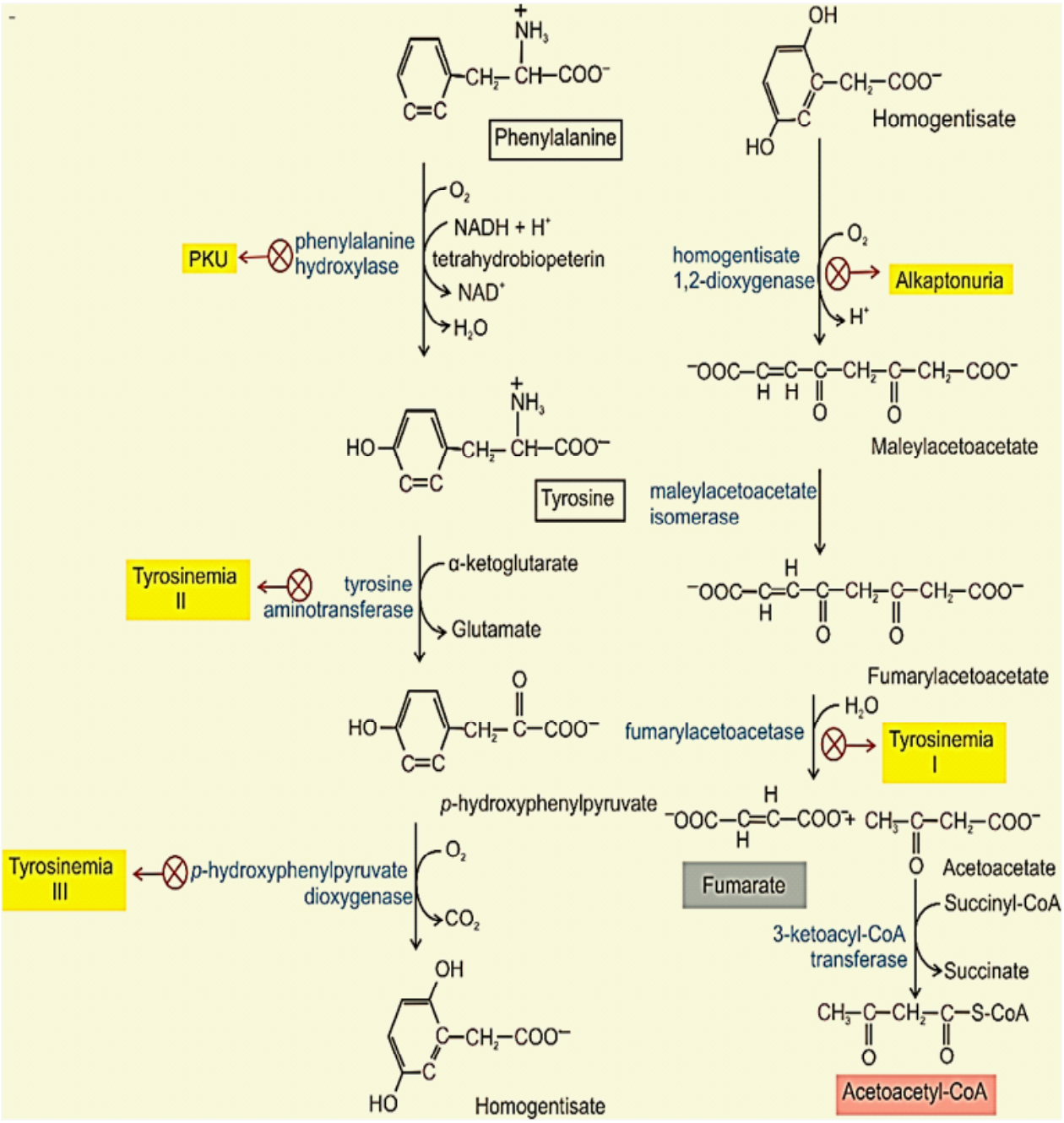

Figure 1.3 Inherited enzyme defects in catabolic pathway of phenylalanine and Tyrosine and the defective enzyme in indicated by ⊗ red summing junctions and the metabolic diseases shaded in the Yellow color (Taken from Principle of Biochemistry, Nelson & Cox, 2004).

Cause

The primary cause of PKU is deficient of phenylalanine hydroxylase, the first enzyme of phenylalanine catabolism which converts phenylalanine into tyrosine in cells (Fig. 1.4). With the deficiency, phenylalanine cannot convert to tyrosine resulting in accumulation of phenylalanine in tissues [7].

A secondary pathway (normally little used) involved in decreasing the concentration of phenylalanine in which it converts to phenylpyruvate which further is reduced or oxidized to form phenyllactate and phenylacetate respectively. Both phenylalanine and phenylacetate accumulate in tissues and blood which can excrete in urine. Hence, the name phenylketonuria.

The characteristic musty odor of urine is due to phenylacetate, which raises suspicion during infancy [8].

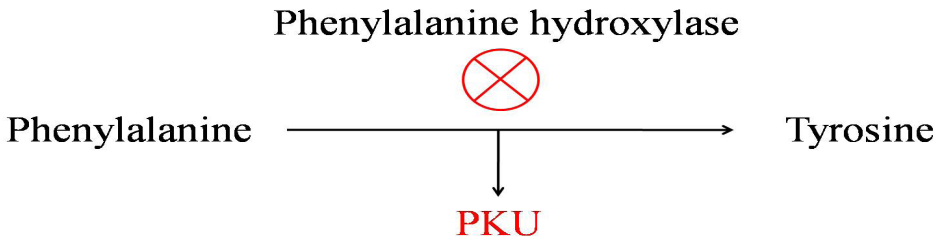

Figure 1.4

Clinical symptoms

Without effective therapy, affected people with PAH deficiency, known as classic PKU, develop profound and irreversible biochemical abnormalities such as mental retardation and neurological dysfunctions, eczema in the early life of infants. Few babies may exhibit epilepsy, Parkinson like features and decreased skin and hair pigmentation.

Diagnosis

The estimation of phenylalanine level in the blood (usually above 600 µmol/L) is primarily used to detect PKU. Increased level of Phenylalanine and phenylpyruvate in blood and urine are analyzed to confirm the PKU using Gas chromatography-mass spectrometry

Treatment

Early diagnosis of PKU in affected person is beneficial for the treatment. People with PKU may recommend restricting the intake of phenylalanine in diet for reducing the toxic effects of phenylalanine accumulation and maintain the level of phenylalanine (2-6 mg/dL) in plasma. The adjuvant therapy with sapropterin is also helpful for PKU treatment.

4.2 Alkaptonuria

Alkaptonuria is the first inborn errors of metabolism discovered by Garrod. Estimated incident of alkaptonuria is about 2-5 per million live births.

Cause

Affected people with alkaptonuria have a deficient enzyme activity of the homogentisate 1,2-dioxigenase. This enzyme metabolizes homogentisic acid to maleylacetoacetic acid (Fig. 1.5).

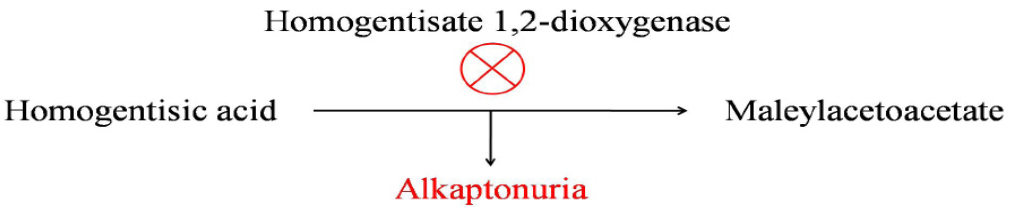

Figure 1.5

Deficient enzyme leads to abnormal accumulation of homogentisic acid in blood and tissues. High level of homogentisic acid in tissues cause a syndrome is called ochronosis. Recent findings reported that alkaptonuria is now considered a multisystemic disease starting from the third decade of life and classified as a secondary amyloidosis [9,10]. On exposure to atmospheric oxygen, urine homogentisic acid is converted into coloured compound [11] (Fig. 1.6).

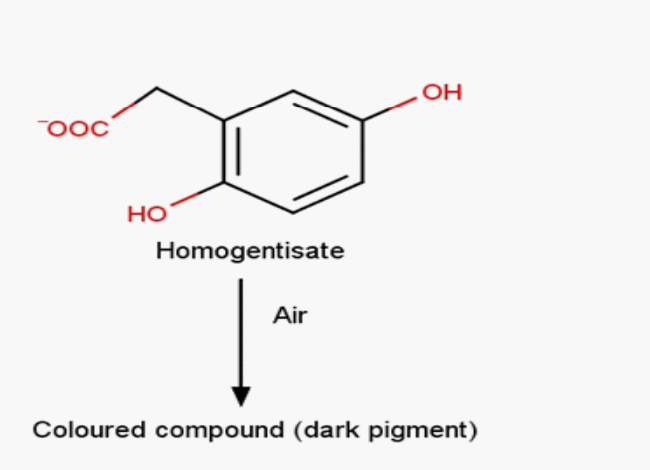

Figure 1.6

Clinical symptoms

Affected person show an abnormal level of homogentisic acid in cartilage tissue caused inflammation and arthritis in older people (Fig. 1.7) [12].

Diagnosis

The urine level of homogentisic acid is primarily measured to the diagnosis of alkaptonuria. The excretion level of HGA is usually about 1-8 grams per day in alkaptonuria's patients.

Treatment

Vitamin C and low proteins diet are recommended to control of the ochronosis by reducing the level of homogentisic acid in tissues. Newborn screening and oral nitisinone therapy may also helpful for the treatment of this disease

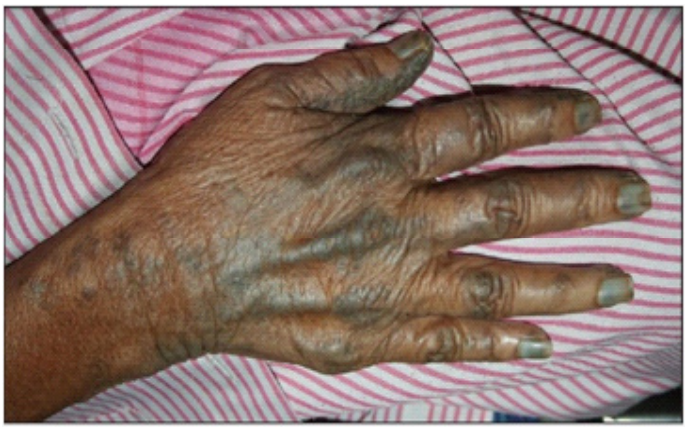

Figure 1.7: Nails and dorsum of hands showing bluish-colored discolorationin affected people with alkaptonuria [13].

4.3 Tyrosinemia

Tyrosinemia is also metabolic genetic disorders of phenylalanine catabolism, occur usually in newborns. This disorder results due to the absence or deficiency of enzymes involved in the multiple steps of phenylalanine and tyrosine catabolism (Refer Fig. 1.4). Untreated tyrosinemia can be fatal for life [14].

However, there are three subtypes of Tyrosinemia:

Tyrosinemia I

Lack of fumarylacetoacetate hydrolase (FAH) enzyme with inherited genetic defect results in tyrosinemia disease. This enzyme involves in tyrosine metabolism which converts fumaryl acetoacetic acid into fumaric and acetoacetic acids (Fig. 1.8). Deficiency of this enzyme, fumaryl acetoacetic acid and other intermediate precursors accumulate in the tissue and organ cause liver and renal diseases. Hence, it is also called hepatorenal tyrosinemia.

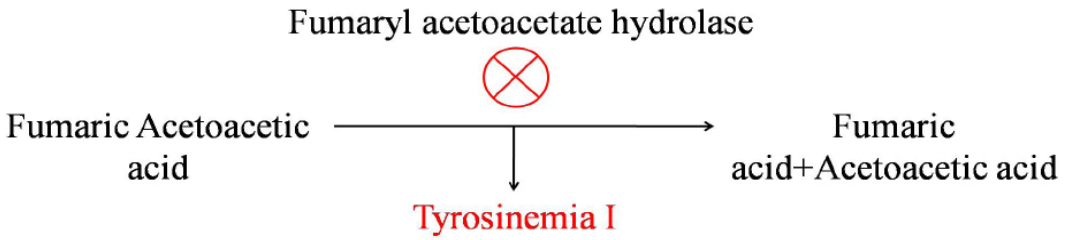

Figure 1.8

Clinical symptoms

Diarrhoea, vomiting, renal tubular dysfunction, vitamin D-resistant rickets, acute intermittent porphyria-like symptoms (abdominal pain, neuropsychiatric findings and sensitive to light), hypertension, Progressive liver and renal failure.

Tyrosinemia II

This disorder is caused by the deficiency of tyrosine aminotransferase enzyme, which catalyzes the first step in the catabolism of tyrosine forming the corresponding keto acid, p-hydroxyphenyl pyruvic acid (Fig. 1.9). Deficiency of this enzyme leads to accumulation of the tyrosine in cells and blood.

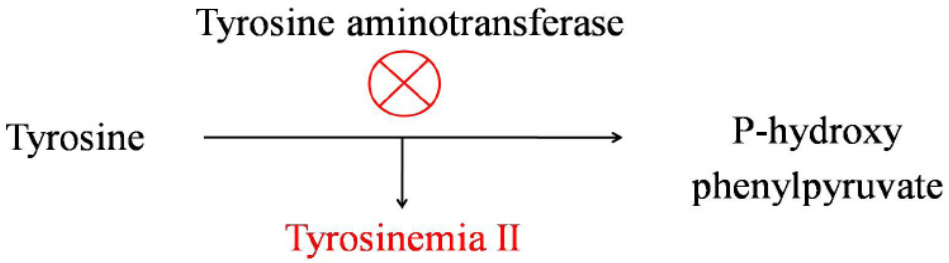

Figure 1.9

Clinical symptoms

Accumulation of tyrosine can affect on eyes, skin, and mental development. This disease begins in early childhood. Persistent keratitis and hyperkeratosis occur on the fingers, palms of hands and soles of feet, moderate mental retardation.

Neonatal tyrosinemia

Tyrosinemia Type III

This disorder occurs due to the defective enzyme, p-hydroxyphenyl pyruvic hydroxylase which normally involved in catalyzing of p-hydroxyphenyl pyruvic acid into homogentisic acid (see Fig. 2.0). The condition is more common in premature infants.

Diagnosis

Estimation of succinylacetone and tyrosine metabolites in blood and urine establishes the definitive diagnosis of Tyrosinemia I and distinguishes from other types of Tyrosinemias.

Treatment

The dietary restriction of tyrosine and phenylalanine with low protein diet may useful to control clinical symptoms of all three types of tyrosinemias. The drug, nitisinone known as NTBC has shown to be effective for the treatment of Tyrosinemia I.

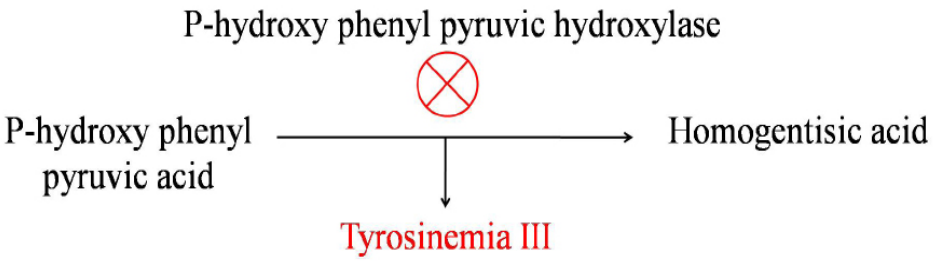

Figure 2.0

4.4 Albinism

Albinism is another congenital hereditary disorder of amino acid metabolism in which biosynthesis of melanin is defective. Melanin is a color pigment absent in certain parts of the body such as eyes, patches of skin and areas of hair. Normally, melanin is polymers of the amino acid tyrosine which gives color to skin, hair and eyes [15].

Cause

Albinism is caused by the mutation in a gene coding tyrosine hydroxylase enzyme. This enzyme converts tyrosine to 3,4-dihydroxy phenylalanine (DOPA) (Fig. 2.1). Deficient activity of this enzyme leads to albinism in which melanin formation is missing. This condition is referred to hypomelanosis [16]. The pale skin, pinkish eyes and visual abnormalities are primary symptoms of this disease.

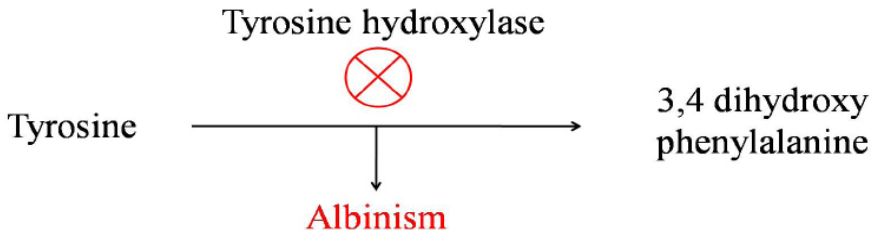

Figure 2.1

Clinical symptoms

Albinism can affect eye and skin in infants or people. This condition refers to oculocutaneous albinism (OCA) resulting in hypopigmentation of the hair, skin and eyes. Therefore, this disease leads to extremely pale skin, poor vision and white hair [17].

Diagnosis

Diagnosis is based on biochemical finding of hypopigmentation of the skin and hair. Molecular genetic testing of OCA gene is available for the albinism diagnosis.

Treatment

There is no treatment for albinism. Albino people require visual rehabilitation such as wear prescription lenses for correction of refractive errors, use hats with brims and dark glasses or transition lenses to reduce discomfort from bright light and wear protective clothing to protect skin from sun exposure

4.5 Urea cycle defects

This is an autosomal recessive inherited genetic disorder that affecting the urea cycle. The liver is a vital organ which plays a promising role in detoxification of nitrogenous wastes

by forming the compound urea through the urea cycle. Urea is a major disposal byproduct of amino acids. The disturbance in the normal urea cycle leads to accumulation of urea causing cellular toxicity (Fig. 2.2). The estimated incidence of urea cycle defects is about 1 in 8,000 live births and generally occurs in the first few days of life.

Cause

The urea cycle disorders (UCDs) are caused by defective or total absence of catalytic activity of the first five enzymes involved in the urea cycle [18]. Errors in this cycle, body unable to detoxify nitrogen content leads to abnormal accumulation of ammonia and other precursor metabolites. These enzymes are:

- Carbamoylphosphate synthetase I deficiency (CPS1)
- Ornithine transcarbamylase deficiency (OTC)
- Argininosuccinic acid synthetase deficiency (ASS1)
- Argininosuccinic acid lyase deficiency (ASL)
- Arginase deficiency (ARG)

A defect in the arginase enzyme resulting argenimia while absence of argininosuccinase and carbamoyl phosphate synthase-I may also cause argininosuccinic acidemia and carbamoyl phosphate I deficiency respectively [19].

Clinical symptoms

Infants with a severe urea cycle disorder are normal at birth but rapidly develop hyperammonemia condition resulting in neurologic damage, coma with presenting symptoms such as cerebral edema, lethargy, anorexia, hyper- or hypoventilation, hypothermia, seizures, neurologic posturing, long term hyperammonemia is toxic to human beings resulting in mental retardation.

Diagnosis

Measurement the elevated level of ammonia, arginine, arginosuccinate in plasma and orotic acids in urine are used to diagnosis of urea cycle disorders

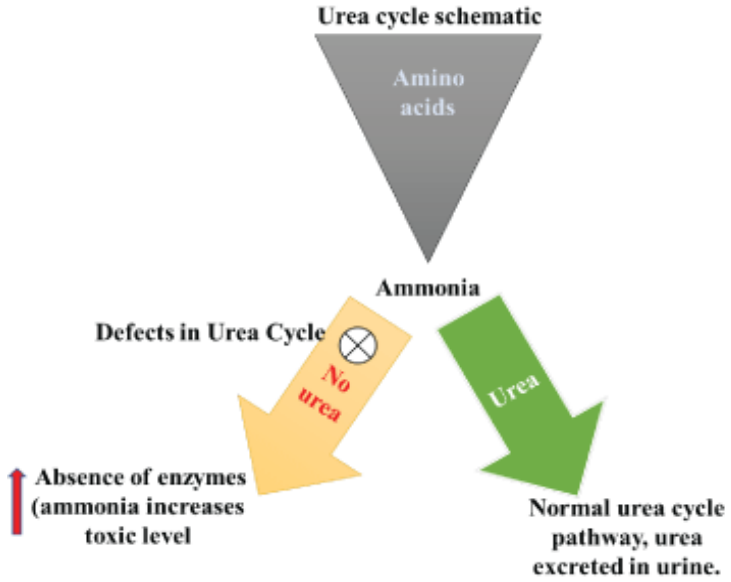

Figure 2.2 Urea Cycle defects

Treatment

Nutritional modification with low protein diet may help in controlling the level of ammonia in the body [20]. Sodium phenylbutyrate is the primary medication used to treat urea cycle disorders. This drug allows an alternative pathway to disposal of nitrogen from the body. If medicine and nutritional treatment failed, liver transplantation becomes an option for UCD's patient.

4.6 Homocystinuria

Homocystinuria is a rare inherited disorder of metabolism of the methionine.

Cause

This disorder results due to the deficit activity or absence of cystathionine β-synthase enzyme involved in methionine degradation. In normal metabolism, methionine converts into homocysteine which further form cystathionine in presence of cystathionine β-synthase as shown in Fig. 2.3. This defect leads to accumulation of homocysteine in tissues [21].

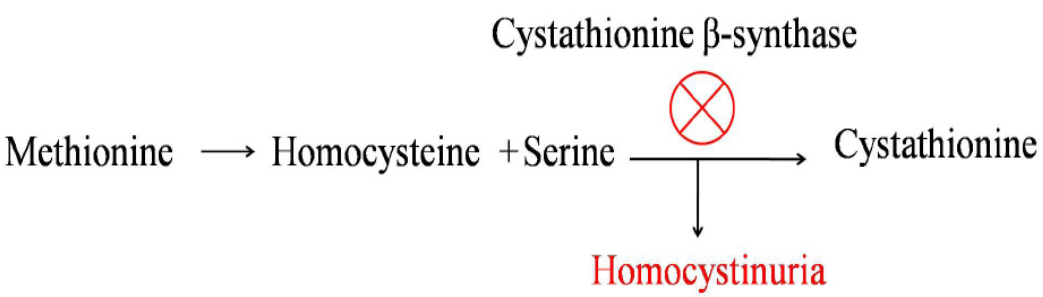

Figure 2.3

Clinical symptoms

High level of homocysteine in cells causing lipid peroxidation, fibrosis, and atherogenesis and affecting muscles, cardiovascular system and nervous system.

Diagnosis

Estimation of the level of homocysteine, total homocysteine, homocysteine-cysteine mixed disulfide, and methioninein plasma.

Treatment

Vitamin B_6 (Pyridoxine) therapy, betaine, folate and vitamin B_{12} supplementation are used to control the biochemical abnormalities, especially to management the plasma homocysteine and homocysteine concentrations and prevent thrombosis.

4.7 Maple syrup urine disease (MSUD)

MSUD is an inherited disorder of branched chain amino acids. Affected people with MSUD have a defective gene inherited from their family.

Cause

The metabolic deficiency of branched-chain enzyme, α-keto acid dehydrogenase causes MSUD in which the body is unable to breakdown the branched-chain amino acids (BCAAs)-Leucine, isoleucine and valine. This leads to buildup keto-acids in tissues and blood resulting metabolic problem. MSUD is also called branched-chain ketoaciduria [22] (Fig 2.4).

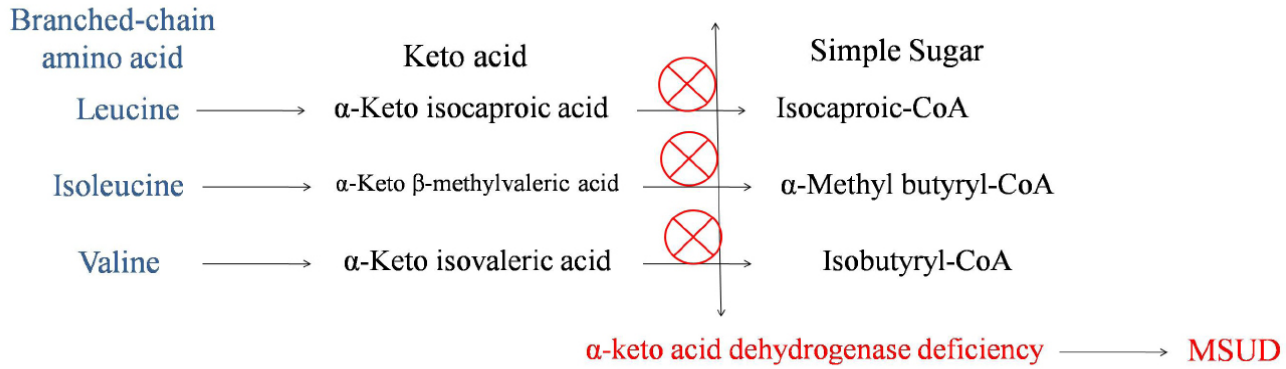

Figure 2.4

Clinical symptoms

MSUD is characterized by the sweet-smelling urine, with an odor similar to that of maple syrup. The biochemical features include neurological disorder, poor feeding, vomiting, dehydration, lethargy, hypotonia, ketoacidosis, hyperammonemia, pancreatitis, and coma.

Diagnosis

Biochemical estimation of plasma levels of BCAAs, allo-isoleucine and the urine levels of branched-chain hydroxyacids and keto-acids (BCKAs) are primary used diagnostic markers for detection of the MSUD.

Table 1.1: Genetic disorders affecting amino acid catabolism

Name of Disease	Defective process	Defective enzyme	Symptoms and clinical effects
Phenylketonuria	Phenylalanine to tyrosine	Phenylalanine hydroxylase	Vomiting and mental retardation
Alkaptonuria	Tyrosine degradation	Homogentisate 1,2- dioxygenase	Dark pigment in urine; acute arthritis
Tyrosinemia	Tyrosine degradation	fumarylacetoacetate hydrolase, tyrosine aminotransferase, p-hydroxyphenyl pyruvic hydroxylase	Diarrhoea, vomiting, renal tubular dysfunction, mental retardation
Albinism	Melanin synthesis	Tyrosine 3-monooxygenase (tyrosinase)	Lack of pigmentation: pale skin, poor vision and white hair
Argininemia	Urea synthesis	Arginase	Mental retardation Vomiting; convulsions Lethargy; convulsions; early death
Argininosuccinic Aciduria	Urea synthesis	Argininosuccinase	
Carbamoyl phosphate Synthetase I deficiency	Urea synthesis	Carbamoyl phosphate synthetase I	
Homocystinuria	Methionine degradation	Cystathionine β-synthase	Faulty bone development; mental retardation,fibrosis, and atherogenesis
Maple syrup urine disease (branched-chain ketoaciduria)	Isoleucine, leucine, and valine degradation	Branched-chain α-keto acid dehydrogenase	Neurological problems, poor feeding, vomiting, dehydration, lethargy, Vomiting

Treatment

Dietary leucine restriction, judicious supplementation with isoleucine and valine, and frequent clinical and biochemical monitoring may help to manage people with MSUD.

5. Disorders of carbohydrate metabolism

The disorders in this group show the wide range of clinical symptoms because of abnormalities in carbohydrate metabolism. Carbohydrates are the main component of our diet

which are made up of long chains of simple sugar molecules. Normally cellular enzymes metabolize carbohydrates into glucose (a type of sugar) or simpler molecules. If an enzyme needs to metabolize of certain sugar is missing, the sugar can accumulate in the body, causing health problems. Carbohydrate metabolic disorders result due to the defect in one or more enzymes involved in carbohydrate metabolism. Let us study about carbohydrate metabolic disorders in this section.

5.1 Galactosemia

The term "Galactosemia" means high galactose level in the blood. It is a rare inherited genetic disorder of carbohydrate metabolismin which the body unable to metabolize the galactose leading to metabolic abnormalities. Although, galactose is natural component found in milk and milk products. The incidence of this disease is about 1 in 18,000 live births.

Cause

People with galactosemia have an inherited defective gene that code galactose-1-phosphate uridyl transferase enzyme. This enzyme converts galactose-1-phosphate to glucose-1-phosphate (Fig 2.5). Some other inherited defective enzymes such as galactokinase and epimerase involved in the galactose metabolism can cause galactosemia disease. Hence, galactose accumulates in the cells which further transport into blood leading to galactosemia [23].

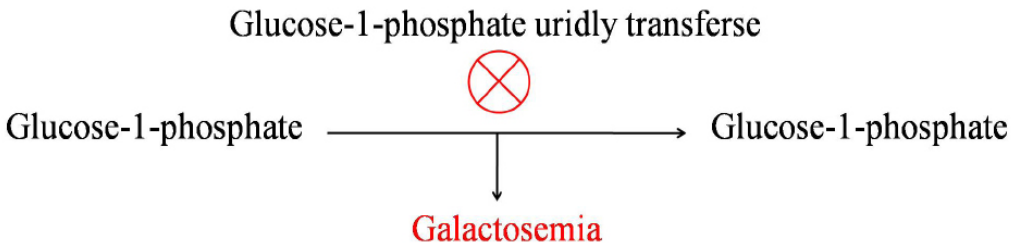

Figure 2.5

Clinical symptoms

Convulsions, irritability, lethargy, poor feeding, poor weight gain, yellow skin and whites of the eyes (jaundice) and vomiting are common symptoms.

Diagnosis

Enzymatic essay of galactose-1-phosphate uridylyl-transferase in the blood is used to detect of affected people with galactosemia. Newborn screening can be done on total galactose levels in blood sample.

Treatment

The clinical symptoms of galactosemia can control with nutritional therapy mainly both galactose and lactose free diet. Infants should be fed a formula (e.g., soy formula) that contains trace levels of galactose or lactose. Continued dietary restriction of dairy products in older children is recommended for galactosemia.

5.2 Hereditary fructose intolerance (HFI)

Hereditary Fructose Intolerance (HFI) is another autosomal recessive genetic disorder of carbohydrate metabolism. Fructose is a natural monosaccharide found in many fruits, vegetables and honey.

Cause

The Acatalytic deficiency of an aldolase-B enzyme (fructose-1,6-bisphosphate aldolase) causes the HFI disease. This enzyme normally converts fructose-1-phosphate to dihydroxyacetone phosphate and glyceraldehyde in glycolysis pathway (Fig. 2.6). Deficiency of this enzyme allows accumulation of fructose-1-phosphate in the liver, kidney and small intestine resulting in metabolic inhibition of glycogen and glucose, thereby causing severe hypoglycemic condition (low sugar in the blood) in the human body.

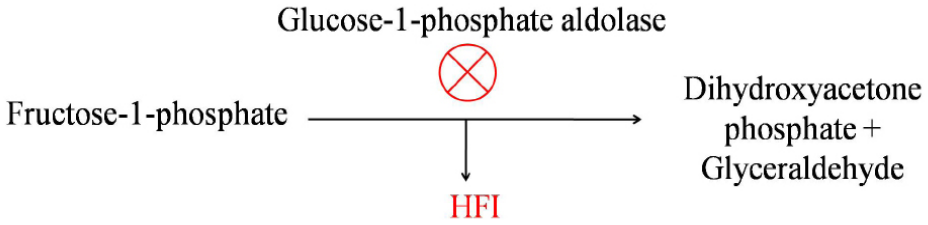

Figure 2.6

Clinical symptoms

Affected people with this disease have symptoms like abdominal pain, vomiting and weakness.

Diagnosis

The direct detection of catalytic activity of Aldolase B enzyme in liver and kidney tissue is useful for diagnosis of HFI. Recently, the diagnosis of HFI has made simpler by the PCR-based method for detection of mutated aldolase-B gene in patients [24].

Treatment

Nutritional treatment along with fructose-free diet may help to manage biochemical symptoms of HFI.

5.3 Hereditary lactose intolerance (HLI)

Lactose intolerance is a common inherited disease in childhood. Lactose, a milk sugar is metabolized by the lactase enzyme in the body. It is an intestinal enzyme which splits dietary lactose into glucose and galactose during the process of digestion (Fig. 2.7). Lactose intolerance can exhibit in children at age three [25].

Cause

Individuals with lactose intoleranceis unable to digest milk and milk product due to lack or insufficient amounts of the lactase enzyme in the body. Normally, lactase breaks down lactose into glucose and galactose. With this insufficiency, lactose accumulates in the intestine wherein intestinal bacteria fermented the lactose by converting it into short-chain acids and gases like hydrogen (H_2) and carbon dioxide (CO_2) which leads to flatulence or stomach pain.

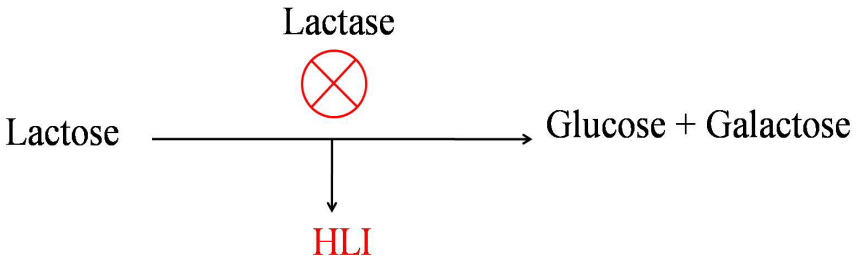

Figure 2.7

Clinical symptoms

Abdominal cramps, diarrhea, flatulence. production of gases like hydrogen (H_2) and carbon-dioxide (CO_2) are common symptoms of lactose-intolerance which leads to intestinal irritants.

Diagnosis

The direct biochemical assay of lactase activity from a jejunal sample is the most common method for the diagnosis of lactose intolerance. Molecular technique is used to identify the defective lactase gene for early diagnosis [26].

Treatment

Treatment requires both fructose and lactose-free dietand limited intake of dairy products recommended for patients.

Table 1.2: Metabolic Disorders of Carbohydrate Metabolism.

Name of Disease	Substrate involved	Enzyme defect	Metabolite accumulated	Reported incidence
1. Galactosemia	Galactose	Galactose-1-phosphate uridyl transferase	Galactose-1-phosphate galactiol	1:50,000
2. Hereditary Fructose Intolerance (HFI)	Fructose	Hepatic aldolase B	Fructose-1-phosphate	1:40,000
3. Hereditary Lactose Intolerance (HLI)	Lactose	Lactose	lactose	-
4. Pentosuria	L-Xylulose	NADP-dependent xylitol dehydrogenase	L-Xylulose	1:50,000
5. Fructosuria	Fructose	Hepatic fructokinase	Fructose	1:130,000

5.4 Glycogen storage diseases (GSD)

A genetic defect in catabolic pathway of glycogen leading to develop glycogen storage diseases. Recall the structure of glucose and glycogen to understand the glycogen storage diseases. Fig. 2.8 showed the structure of glycogen consists of branched-chain polymers of glucose residues (up to 50,000) and the residues link together within chain by glycosidic bonds (α-1 → 4 and α-1 →6). Glycogen is a storage form of glucose stored mainly in the liver and muscles.

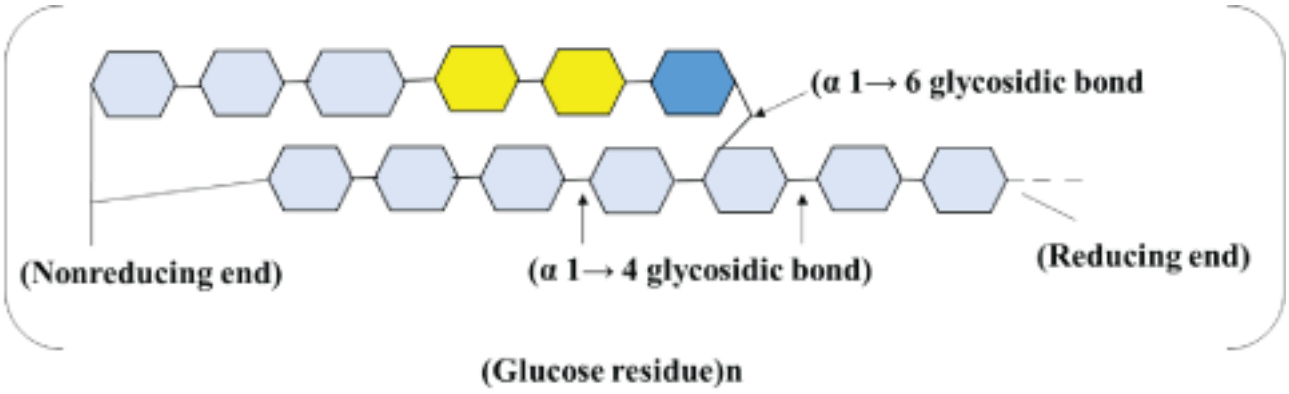

Figure 2.8 Structure of glycogen

When blood sugar level goes down, glycogen is broken down into glucose which furthers transports into the blood. Deficiency of enzymes involved in the glycogen metabolism result in progressive accumulation of glycogen in the liver and muscles. The most affected organ is the liver [27].

Let us discuss important glycogen storage diseases:

5.4.1 Glycogen storage disease type I (GSD-I) or von-gierke disease

In 1929, Von Gierke described the first glycogen storage disease. It is also known as Von Gierke disease.

Cause

An inherited defect in the glucose-6-phosphatase enzyme causes metabolic abnormalities in glycogen metabolism. This enzyme catalyzes glucose-6-phosphate to glucose in liver and muscles. This enzymatic defect leads to developing Von Gierke disease in which glycogen cannot convert finally into glucose molecules (Fig. 2.9). This disease is characterized by low blood sugar level and excessive accumulation of glycogen in liver and muscle. Due to absence of this enzyme,

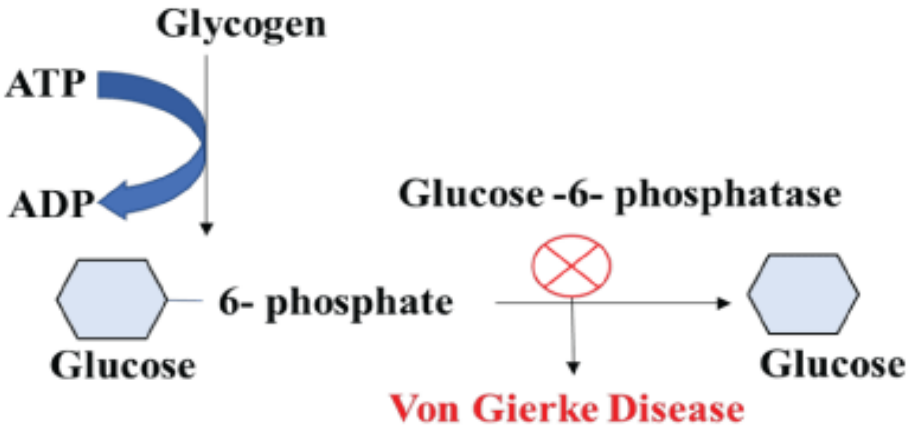

Figure 2.9

Clinical symptoms

The primary symptom in untreated infants is hypoglycemia, hepatomegaly, lactic acidosis, hyperuricemia, hyperlipidemia, hypoglycemic seizures. Progessive complications are fatty liver, doll-like faces, short stature and protuberant abdomen are observed in affected people and children [28].

Diagnosis

Histological examination of liver tissues can be used to visualize abnormal glycogen content in affected people with type I disease. The detection of GSD-I gene in patients can allow for early diagnosis of this disease.

Treatment

Nutritional therapy may help to maintain blood glucose levels, to control hypoglycemia, and to provide optimal nutrition for growth and development. The nutritional interventions include frequent daytime feedings, nighttime intragastric continuous glucose infusion and oral uncooked cornstarch may necessary for the management of this disease. Liver function test must be monitored for the efficacy of dietary treatments.

5.4.2 Pompe disease or Type II glycogen storage disease

It is an autosomal recessive inheritance disorder caused by mutation in the gene coding, acid-α-glucosidase enzyme. It is also commonly shortened to GAA that stands for glucosidase acid alpha, which is the name given to the gene. This GAA gene active in lysosomes of liver, heart and muscle tissues. This disease occurs in infant, children and people who inherit a defective gene from their parents. This disease is also referred to as lysosomal storage disease.

Cause

People with the pompe disease have a deficiency of acid-α-glucosidase enzyme (Fig. 3.0). With this genetic disorder, the body cannot breakdown glycogen into the simple sugar (glucose) leading to accumulation of lysosomal glycogen in the heart, skeletal and smooth muscle, and the nervous system [29].

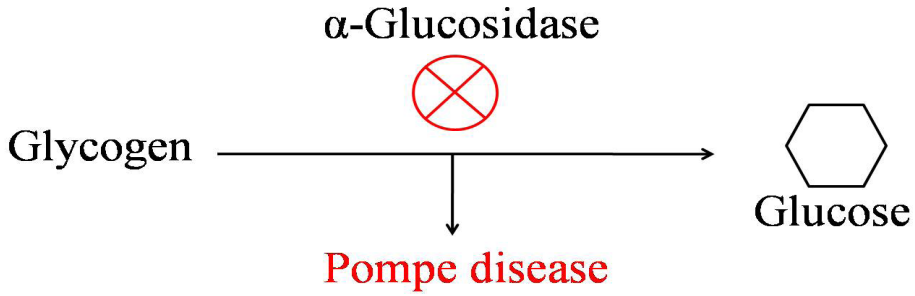

Figure 3.0

Clinical symptoms

Classical infantile-onset pompe disease symptoms can appear in infants with muscle weakness, breathing problems, feeding problems leading to failure of respiratory system and hearing loss. Without treatment or delay diagnosis, this disease can be harmful to health of the infants or children.

Diagnosis

Enzymatic assay of GAA activity in blood is the common way to confirm the Pompe disease in patient. GAA mutation analysis is confirmatory test for diagnosis of this diseases.

Treatment

Nutritional therapy may provide temporary improvement in patient with Pompe disease but early diagnosis is better option for the treatment. Enzyme replacement therapy (ERT) with Myozyme® or Lumizyme® (alglucosidase alfa) can become new treatment options for affected people with the Pompe disease [30].

5.4.3 Type III or Cori disease

Glycogen storage disease Type III is known as Cori disease. It is an inherited, an autosomal recessive genetic disorder caused by a mutation in the gene coding debranching enzyme "amylo (α-1→6) glucosidase" which is a key enzyme to split branched-glucose molecules from glycogen in glycogen degradation. The conversion of glucose to lactate in the muscles and lactate to glucose in the liver is called the Cori cycle [31].

Cause

People who lack the debranching enzyme leading to develop the Cori disease. This diseases causes progressive accumulation of glycogen which impaired the physiological functions of organ and tissues particularly, in liver and muscle (Fig. 3.1).

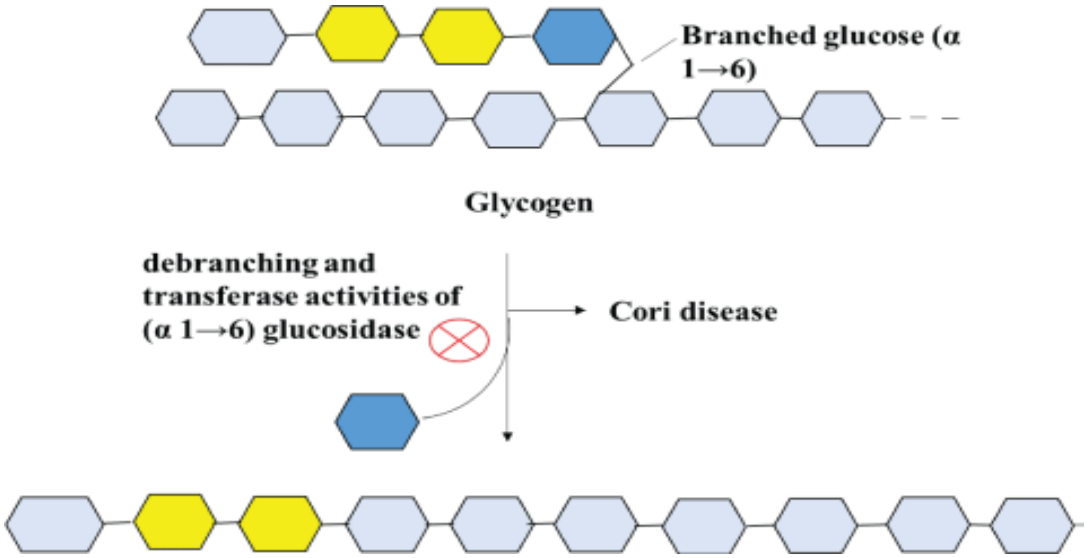

Figure 3.1

Clinical symptoms

The dominant clinical features of this disease are hepatomegaly, hypoglycemia with fasting and its clinical significance ranges from asymptomatic in the majority to severe cardiac dysfunction, congestive heart failure, and (rarely) sudden death [32].

Diagnosis

Elevated serum concentrations of transaminases and CK are the hallmarks of GSD III. The debranching enzyme activity test and histopathology examination are useful for the definitive diagnosis of Cori diseases.

Treatment

GSD III's patients treated with high protein diet to control the clinical symptoms.Currently, there is no effective treatment for progressive hepatomegaly

6. Disorders of lipid metabolism

Inherited genetic defects in the lipid metabolism leads to excess storage of lipids in ectopic tissues, such as skeletal muscle, liver, and heart, seems to associate closely with metabolic abnormalities and cardiac disease. Storage disorders involve in specific enzyme deficiency in lysosomal degradation pathways. In fact, most lipid metabolic disorders arise from inherited defect in the catabolism of sphingolipids. The lipid-like sphingolipid is a constituent of cell membrane primarily rich in brain cells. Fig. 3.2 shows some disorders in degradative pathway of membrane lipid sphingolipids and inherited defective enzymes.

Let us discuss biologically important lipid metabolic disorders.

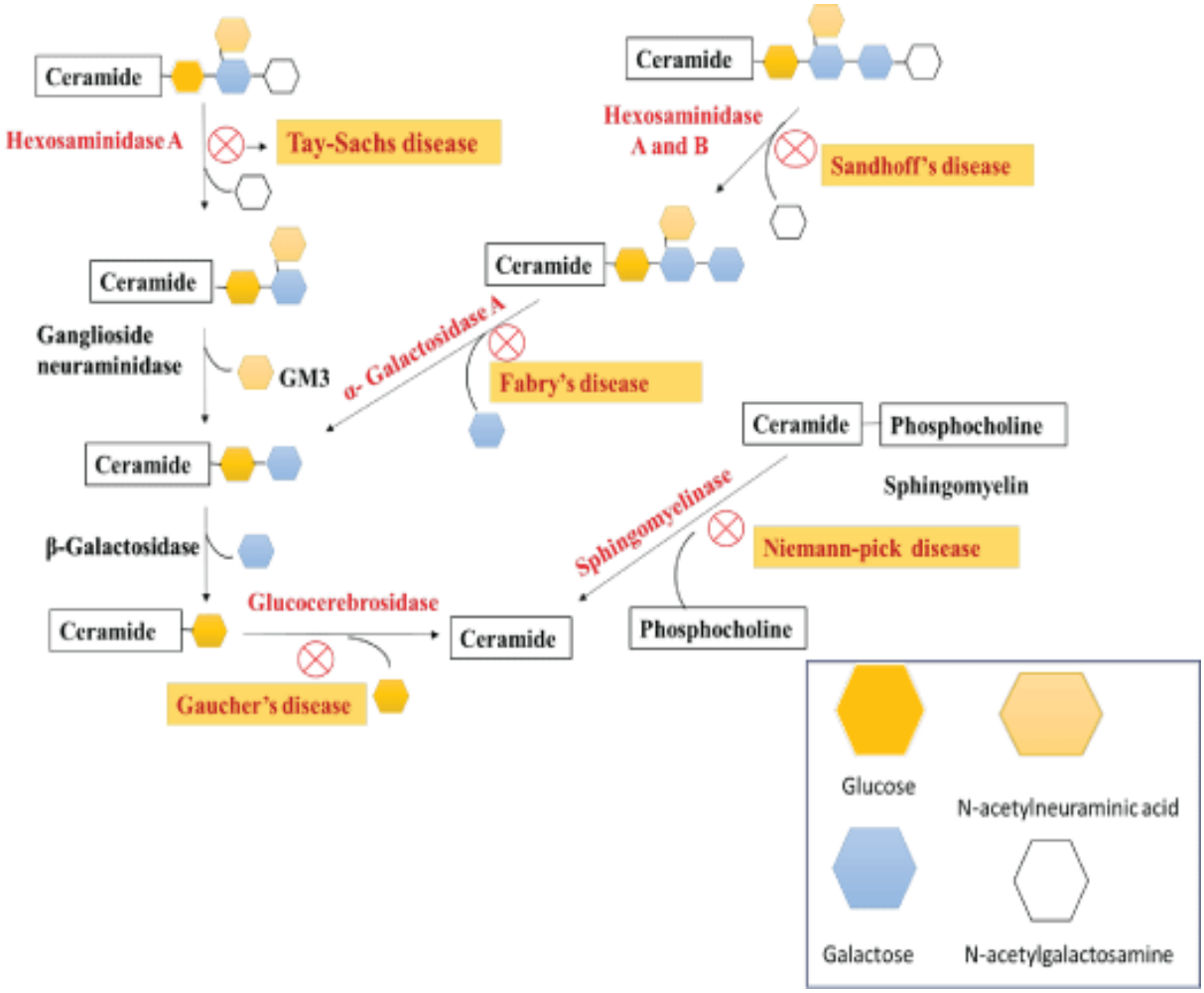

Figure 3.2: Metabolic defects in the Sphingolipidsmetabolism (Courtesy: Nelson & Cox, 2008).

6.1 Gaucher disease (Glucosyl ceramide lipidosis)

In 1882, the French physician Philippe Gaucher described the first lipid storage disease in lysosomes and hence the name is referred as Gaucher Disease. Overall, the estimated incidence is about 1 in 7000 live births.

Cause

Glucocerebroside metabolism is caused by the β-glucocerebrosidase enzyme deficiency, which normally breakdowns glucocerebroside into glucose and ceramide (Fig. 3.3). With the

deficiency, glucocerebroside accumulates in lysosomes and macrophages resulting in enlargement of cells. These abnormal cells are called Gaucher cells [35].

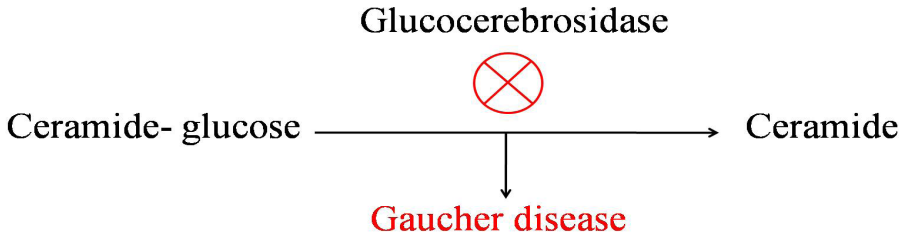

Figure 3.3

Clinical symptoms

The common features of this disease are osteopenia, sclerotic lesions, hepatosplenomegaly, anemia, thrombocytopenia and lung disease which may finally affect in proper functioning of central and peripheral nervous system.

Diagnosis

The test for determination of deficient activity glucocerebrosidase enzyme in the blood and analysis of β-glucocerebrosidase enzyme gene may reliable for the diagnosis.

Treatment

Enzyme replacement therapy (ERT) or substrate reduction therapy (SRT) is available as the first effective treatment for Gaucher disease.

6.2 Fabry disease

Unlike most other sphingolipidoses, Fabrydisease is an inherited X-linked recessive lysosomal storage disorder, occurring mostly in adults. The Johannes Fabryand Anderson discovered Fabry disease in 1998 hence, it is also referred to as Anderson-Fabry disease. The estimated incidence is about 1 in 40,000 live births.

Cause

Genetic mutation in a gene encoding α-galactosidase, a lysosomal enzyme leads to develop Fabry disease (Fig. 3.4). Deficiency or low activity of α-galactosidase-A enzyme causes abnormal accumulation of sphingolipid in lysosomes leading to a wide range of clinical adverse effects including renal, cardiac, cerebrovascular and skin disorders [34].

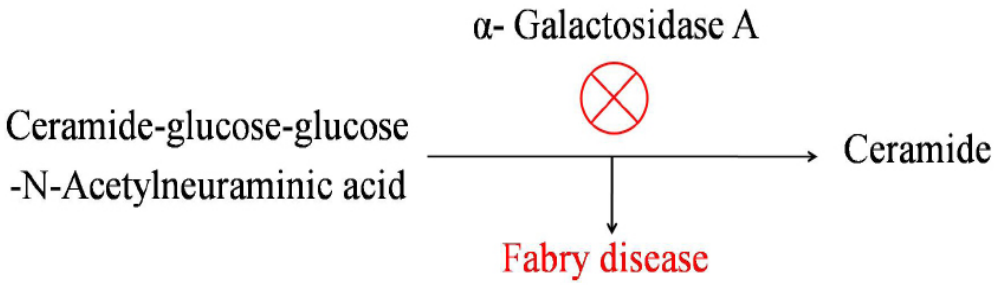

Figure 3.4

Clinical symptoms

If untreated, affected people with Fabry disease have average life expectancy about 15-20 years. The classical symptoms include renal failure, cardiovascular dysfunction, neuropathy, stroke and skin disease.

Diagnosis

Demonstration of marked a-galactosidase A deficiency in blood or tissues is the definitive method for the diagnosis. Molecular analysis of GLA enzyme gene can also be done to diagnosis.

Treatment

Enzyme replacement therapy along with medicine may useful for preventing the symptoms of Fabry disease.

6.3 Niemann-pick disease (Sphingomyelin lipidosis)

Niemann-Pick disease is another lipid storage disorder which was first described by Niemann and Pick. It is also known as sphingomyelin lipidosis. It is an autosomal recessive genetic disorder in which the body unable to metabolize of sphingomyelin in cells. Due to lack of lysosomal sphingomyelinase human cells cannot metabolize sphingomyelin. This stage leads to abnormal storage of sphingomyelin primarily in lysosomes. Hence, it is called lysosomal storage disorders. Lipids like sphingomyelin and cholesterol present in all cell membrane and metabolize in lysosomes [36].

This disease is classified into two types:

Niemann-Pick Type-A disease results in neurological dysfunctions in infants and hepatosplenomegaly.

Niemann-Pick Type-B affects visceral system with little to no neurological detriment.

Cause

Both diseases (Types A & B) are caused by inherited defect in the lysosomal sphingomyelinase enzyme, which normally breakdowns sphingomyelin into phosphorylcholine and ceramide (Fig. 3.5).

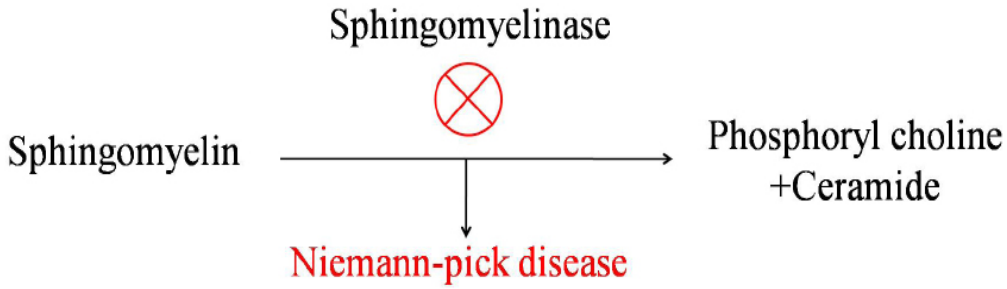

Figure 3.5

Clinical symptoms

Include enlarged liver and spleen, mental retardation, a classic cherry-red spot in the retinaand early death.

Diagnosis

The diagnosis is established by detection of sphingomyelinase enzyme activity in peripheral blood lymphocytes or cultured skin fibroblasts.

Treatment

Adequate nutrition with low- cholesterol dietis recommended for supportive treatment to manage the symptoms of NPC diseases. There is no effective treatment for NPC diseases, but stem cell transplantation may help to cure the Type-A and Type -B diseases.

6.4 Tay-Sach's disease (TSD) (Ganglioside lipidosis)

This disease is an inherited disorder of lipids particularly sphingolipids in which lipids accumulate in the cells leading to health problems. Gangliosides are glycosphingolipids contain a fatty acid, sphingosine, oligosaccharide chain. It is rich in the nerve cell membrane.

Cause

Tay-Sach's disease results from a genetic defect in the hexosaminidase-A enzyme (Hex-A). This is a vital enzyme which cleaves glycosidic bond attaching N-acetyl-hexosamine in ganglioside GM2 (Fig. 3.6). Due to the absence of Hex-A enzyme, GM2 cannot convert to GM1 resulting in accumulation of sphingolipids in brain leading to degeneration of the nervous system [33].

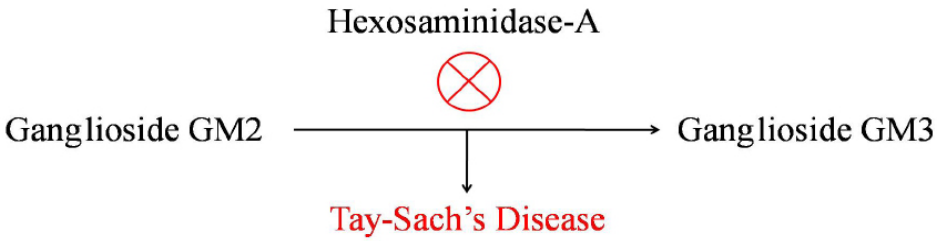

Figure 3.6

Clinical symptoms

The symptoms of Tay-Sachs disease begin between ages three and six months with progressive weakness, growth retardation, loss of motor skills, paralysis, blindness, Without treatment, death, usually by 3 to 4 years. More than 90 % of the patients with this disease have a characteristic cherry-red spot in the retina.

Diagnosis

The determination of hexosaminidase-A enzyme activity (HEXA) in the serum or white blood cells is the common biochemical marker for Tay-Sachs disease. Molecular genetic testing of HEX-A enzyme gene is the best accurate method for diagnosis of Tay-Sach's disease.

Treatment

Affected people with this disease needs to support with adequate nutrition and hydration. Genetic analysis and enzymatic screening programs can help to control this disease.

7. Disorders of purine and pyrimidine metabolism

Inherited defective genes cause enzymatic deficiencies in purine and pyrimidine metabolism. As you know, purine and pyrimidine are nitrogenous bases that form nucleic acids (DNA and RNA). Let us discuss first purine metabolic disorders.

7.1 Lesch-nyhan syndrome

A genetic lack in hypoxanthine-guanine phosphoribosyl transferase (HGPRT) enzyme activity results in Lesch-Nyhan syndrome. Although, it is a rare syndrome caused by a mutation in the *HGPRT* gene located on the X-chromosome. HGPRT involves in the salvage pathway in which purine and pyrimidine are recycled from free purine and pyrimidine nitrogenous bases. This enzyme helps to convert hypoxanthine to inosine monophosphate (IMP) and guanine to guanosine monophosphate (GMP). The final step of this pathway, xanthine oxidase enzyme converts hypoxanthine to xanthine which is further oxidized to uric acid and it excretes primarily in the urine (Fig. 3.7). This syndrome leads to excessive accumulation of uric acid in

cells causing toxicity. The most affected organ is the brain [37].

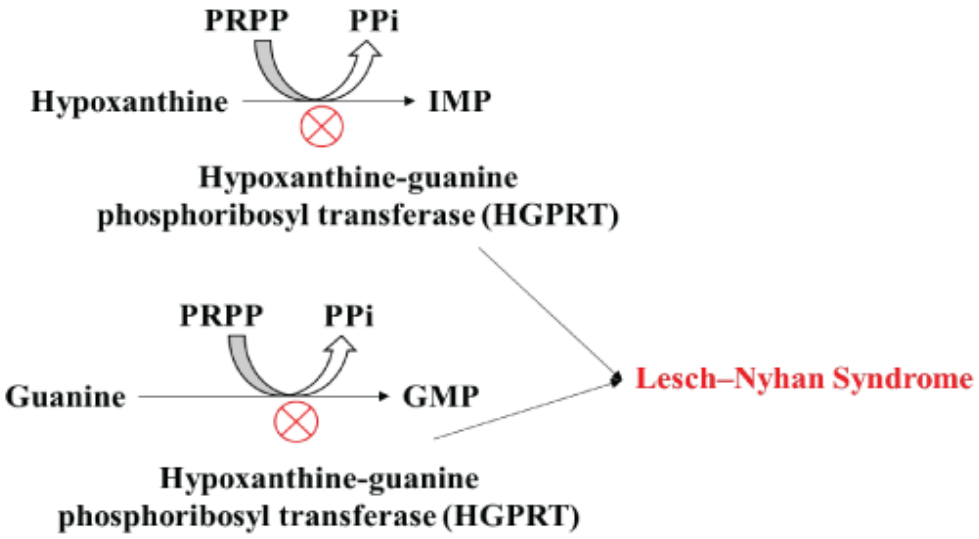

Figure 3.7

7.1.1 HGPRT deficiency leads to gout disease

Gout is a common disorder associated with HGPRT deficiency which is characterized by high level of uric acid in the blood (hyperuricemia). Uric acid is anend-product of the purine degradation. In the blood, most uric acid is present in the form of monosodium urate. High levels of urate deposit in crystal form in tissues resulting skin nodules (tophi), joint pain, kidney damage and stones. The normal level of uric acid is 3-7 mg/dl in blood when its level is more than 7 the condition is called hyperuricemia. Urate crystal saturates in the tissues and joints caused a serious clinical problem.

Clinical symptoms

The most common clinical features include hypotonia and developmental delay are evident by age three to six months in affected children. Persistent self-injurious behavior (biting the fingers, hands, lips, and cheeks; banging the head or limbs) is a hallmark of the disease. Abnormal disposition of urate crystals in joints can cause joint pain and acute arthritis.

Diagnosis

Estimation of high urate concentration and HGPRT enzyme activity in serum are the main diagnostic tool for prompt diagnosis. A urinary urate-to-creatinine ratio greater than 2.0, indicating uric acid overproduction (hyperuricemia), is a characteristic for children younger than age ten years. HPRT1 is the only gene known to be associated with Lesch-Nyhan syndrome.

Treatment

The high rich diet of meat and seafood containing uric acid should be restricted. Allopurinol drug can use to treat people with gout and this drug inhibits xanthine oxidase activity and reduces the formation of uric acid. Non-steroidal anti-inflammatory drug (NSAID) may recommend to reducing the clinical symptoms of the gout disease.

7.2 Adenosine deaminase deficiency

The adenosine deaminase deficiency is known as the Severe Combined Immunodeficiency (SCID). The SCID is an autosomal recessive disorder results from mutation in the gene encoding adenosine deaminase (ADA) enzyme which converts adenosine to inosine in the purine catabolism (Fig. 3.8) [38].

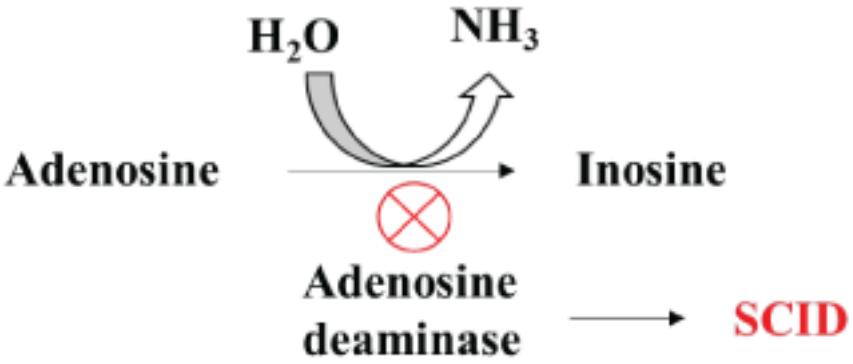

Figure 3.8

This enzyme is present in a variety of cells, mainly lymphocytes cells. ADA deficiency leads to accumulation of adenosine and rises dATP levels resulting in depletion of lymphocytes cells. It also decreases production of T cells, B cells and natural killer cells (NK cells). The incidence of ADA deficiency is approximately 14%. Untreated children with this disorder usually die by the age of two.

Clinical symptoms

Usually present with failure to thrive, opportunistic infections, persistent diarrhea, extensive dermatitis and recurrent pneumonia.

Diagnosis

Estimation of ADA catalytic activity in blood cells confirmed the diagnosis of deficiency of ADA. Molecular genetic analysis of ADA gene can be performed for the ADA deficiency.

Treatment

Bone marrow transplantation is the best option for the treatment of SCID. Bovine ADA has been shown to be beneficial for appropriate therapy for SCID's patients.

7.3 Disorders of pyrimidine metabolism

7.3.1 Orotic aciduria

This is an inherited autosomal recessive disorder caused by a deficiency of UMP synthase, a bifunctional protein includes the enzyme activities of orotate phosphoribosyl transferase and orotidine-5′ phosphate decarboxylase which catalyze orotate to OMP and OMP to UMP respectively (Fig. 3.9). With the deficiency, orotic acid accumulates in tissue and is excreted in urine. Abnormal accumulation of orotic acid leading to significant clinical health problems [39].

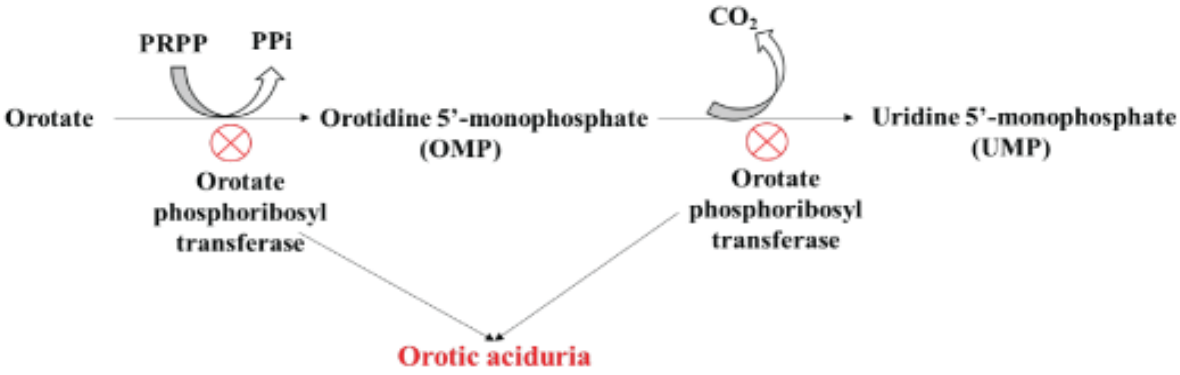

Figure 3.9

Clinical symptoms

Megaloblastic anemia, oroticcrystalluria, nephropathy and cardiac malformations are common clinical features of this disease.

Diagnosis

Gas chromatography-mass spectrometry is used to analyze the level of urinaryorotic acid for detection of this disease.

Treatment

Replacement therapy with uridine usually leads to a clinical and hematologic remission and reduction in the urinary excretion of orotic acid. Allopurinol is useful to reduce orotate level and uric acid excretion in affected people with this disease.

8. Summary

- The inborn errors of Metabolism (IEM) are significantly interrelated with genetic abnormalities. The inheritance pattern of genes may be either dominant or recessive which inherit from carrier parents to their children. In other words, defective genes lead to synthesis of defective enzymes. The basis of EMI is the gene mutation in single genes that code for an enzyme resulting in synthesis of deficiency of a specific enzyme involved in metabolic pathway of amino acids, carbohydrates, lipids, urines, and pyrimidines.

- Absence or deficiency of enzymes in metabolism lead to blockage in individual steps

of normal metabolic pathway resulting in biochemical abnormalities. As a result, intermediate metabolites accumulate in tissues and bold system which consequently affecting the biochemical and physiological functions of the cells and vital organs of the body and its consequences progressive Health problems arise. Clinical symptoms of IEM may appear with few days in affected people or newborns resulting in physical and mental abnormalities in human beings.

- Early diagnosis, genetic analysis of newborns, screening of future parents and nutritional treatment may help in reducing the chance to develop clinical symptoms and to management of the inherited metabolic disorders

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Chapter 4

Microbial Cellulase and Xylanase: Their Sources and Applications

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Abstract

Cellulose and xylan, the two major constituent of lignocellulose are the most abundant and renewable resource available on earth. Cellulose and xylan are complex substrates and their complete hydrolysis requires a variety of enzymes. Cellulases and xylanases are produced by microorganisms, algae, protozoans, crustaceans and insects however, fungal and bacterial bioconversions are economically viable. In the present chapter, remarkable collections of fungi and bacteria have been brought to the limelight that can degrade cellulose and xylan. Mode of action and brief classification of various cellulases and xylanases have been mentioned. Further, insight knowledge on use of cellulases and xylanases for bioremediation and industrial applications were also provided.

Keywords: Lignocellulose; cellulose; xylan; cellulase; xylanase; bacteria; fungi

1. Introduction

The limitation in the availability of fossil fuels and their negative impact on environment made researchers to develop new eco-friendly processes based on renewable feedstock. Lignocellulose is one among such renewable feedstock [1]. Lignocellulose is the major component of plants and referred as in exhaustible natural renewable sources on earth [2]. Lignocellulose mainly consists of cellulose, hemicellulose, and lignin; along with small amounts of pectin, protein, extractives, and ash. However, these compositions vary depending on the plant source, but typically the major part consists of cellulose, followed by hemicellulose, and lignin [3].

Cellulose is an unbranched homopolysaccharide consist of D-glucopyranosyl units [4] Hemicelluloses are branched heteropolysaccharides consist of pentoses (β-D-xylose, α-L-arabinose), hexoses (β-D-mannose, β-D-glucose, α-D-galactose) and/or uronic acids (α-D-glucuronic, α-D-4-O-methylgalacturonic, and α-D-galacturonic acids). Other sugars such as α-L-rhamnose, and α-L-fucose may also be present in small amounts and the hydroxyl groups of sugars can be partially substituted with acetyl groups [5].

Lignin is a highly irregular and insoluble polymer consisting of phenylpropanoid subunits. Unlike cellulose or hemicellulose, no chains containing repeating subunits are present in lignin, thereby making the enzymatic hydrolysis of this polymer extremely difficult [2,4,6]. In order to utilize lignocelluloses chemical or biological hydrolysis are required. Chemical hydrolysis mainly includes acid, alkali and steam explosion. Recent development in biological hydrolysis, it is now known to be more effective, economical, eco-friendly as compared to the chemical-based approaches and hence biological hydrolysis are now replacing the chemical-based treatments [7].

Biological hydrolysis of lignocelluloses is complex and requires multi-enzyme system [8]. Different components of lignocellulose require different enzymes. For the efficient hydrolysis of cellulose, the action of at least three enzymes namely, endoglucanases, exoglucanases and β-glucosidase are required [9]. Xylan hydrolysis (the major part of hemicellulose) needs multi-enzyme systems, such as endoxylanases, β-xylosidases, α-L-arabinofuranosidases, and acetyl esterases [8]. Lignin degradation requires two major groups of enzymes mainly heme-peroxidases and laccases [10]. Since, various enzymes are involved in lignocellulose hydrolysis and it is difficult to include all in a single chapter hence, the present book chapter focus on enzymes involved in the hydrolysis of two major parts of lignocellulose, cellulose and xylan. It lists some fungal and bacterial derived cellulase and xylanase and their possible applications.

2. Cellulases

Cellulases (EC 3.2.1.4) are the enzymes that break the cellulose molecule into monosaccharide or shorter polysaccharides [11]. The degradation of cellulose involved two steps. In the first step, anhydroglucose chains are swollen or hydrated and in the second step, hydrolytic cleavage of susceptible polymers either randomly or endwise occurs [12].

Cellulases have been produced by microorganisms including bacteria, archaea and fungi. Cellulases were also produced by some animals, but their function in animal system are still unclear [13,14].

2.1 Enzymatic hydrolysis of cellulose

The enzymes required for the hydrolysis of cellulose are exoglucanases, endoglucanases, β–glucosidases and cellobiose phosphorylase. Exoglucanases are the type of cellulase that acts on the terminal end of cellulose chain, releasing glucose or cellobiose as the end product (Figure 1). Exoglucanase are of two types; 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91) removes cellobiose units while 1,4-β-D-glucan glucohydrolase (EC 3.2.1.74) removes glucose units [15].

In contrast to exoglucanases, endoglucanases (EC 3.2.1.4) are responsible to initiate cleavage and hydrolyze cellulose randomly at the internal regions, releasing oligosaccharides (Figure 1) [16]. β-Glucosidases (EC 3.2.1.21) catalyze the hydrolysis of β-1-4 bonds linking two glucose or substituted-glucose molecules [16,17]. Cellobiose phosphorylase catalyzes the reversible phosphorolytic cleavage of cellobiose [18]. A list of fungi and bacteria producing different type of cellulase represented in Table 1.

2.2. Microbial source for cellulase

Utilization of cellulose in sufficient amount to provide usable energy to an organism was thought to be carried out by microorganisms [9]. A wide range of microorganisms are capable of producing cellulase. Cellulase production has been reported by both aerobic and anaerobic microorganisms. However, there is a distinct difference in cellulose degradation strategy. Usually, anaerobes degrade cellulose via complex cellulase systems exemplified by the well-characterized polycellulosome organelles. Several anaerobic species that utilize cellulose do not release measurable amounts of extracellular cellulase, and instead have localized their complex cellulases directly on the surface of the cell or the cell-glycocalyx matrix. Aerobic cellulose degraders utilize cellulose through the production of extracellular cellulase enzymes that are freely recoverable from culture supernatants [9].

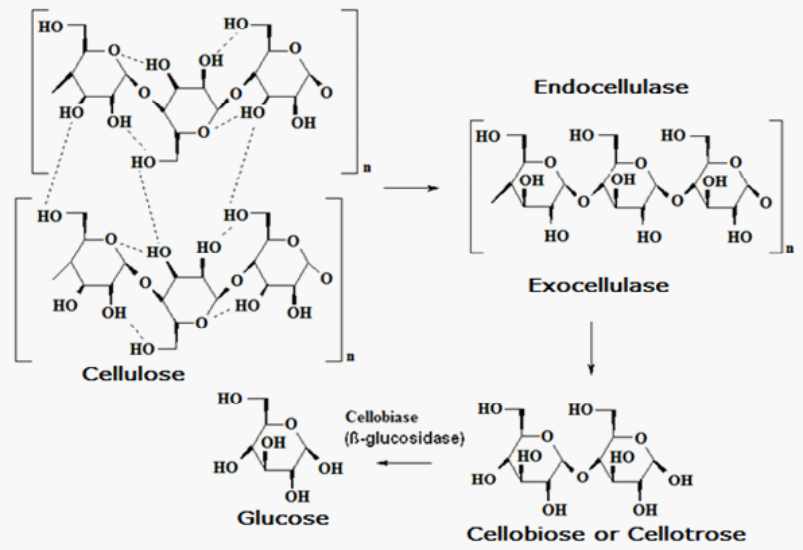

Figure 1: Enzymatic hydrolysis of cellulose.

Table 1: List of fungi and bacteria producing different class of cellulase

Fungi/bacteria	Type of cellulase	References
Aspergillus niger	Exoglucanase	[15]
Cellulomonas flavigena	Exoglucanase	[19]
Bacillus subtilis	Exoglucanase	[11]
Clostridium stercorarium	Exoglucanase	[20]
Irpexlacteus	Exoglucanase	[21]
Gloeophyllum trabeum	Endoglucanase	[22]
Thermoascus aurantiacus	Endoglucanase	[23]
Cellulomonas, Bacillus and *Micrococcus* spp.	Endoglucanase	[24]
Streptomyces misionensis PESB-25	Endoglucanase	[25]
Trichoderma	β – Glucosidases	[26]
Aureobasidium pullulans	β – Glucosidases	[27]
Penicillium decumbens	β – Glucosidases	[28]
Neocallimastix patriciarum	β – Glucosidases	[29]
Ceriporiopsis subvermispora	β – Glucosidases	[30]

2.2.1. Bacterial cellulase

Bacteria producing cellulase have been reported from various ecological niches, such as soil sample [31], dairy farm soil [32], mangrove soil [33], deep-sea sediment [34], hot springs [35], rhinoceros dung [36], pig intestine [37], gut of fish [38], etc. Cellulases have been reported by aerobic, anaerobic and facultative bacteria, Table 2 highlight some of them.

In the past few years, many cellulase producing novel strains (Table 3) including aerobic, anaerobic and facultative were added to the prokaryotes list such as gram negative, aerobic novel genus (of the family Phyllobacteriaceae) isolated from surface seashore water (*Oricolacellulosilytica*) was reported for cellulose degradation [39]. *Clostridium phytofermentans,* an obligate anaerobic novel species, isolated from forest soil was reported for cellulase activity [40]. Novel genus *Cellulosibacter* (type species as *Cellulosibacter alkalithermophilus*) isolated from coconut garden was reported for cellulase activity [41].

Cellulase for industrial applications needs to withstand various extreme conditions such as temperature and pH. Bacterial cellulases have been considered as an important industrial source as they can withstand extreme temperature and pH [42, 43]. For food industry, environmental bioremediation, and molecular biology study psychrophiles cellulase are needed [44]. Psychrophilic cellulase from *Pseudoalteromonas haloplanktis* can be used for such applications [45]. In some steps of textile industry, acidic and psychrophilic cellulases are required. Bacteria are also reported for such cellulase. Bacteria such as *Klebsiella* sp. produce cellu

lase active at 10 °C and pH 4.5, this type of cellulase can be used in textile industries [46]. Apart from psychrophilic cellulase, bacteria also produce alkali and thermo tolerant cellulase. *Marinobacter* sp. (MSI032) isolated from the marine sponge has been reported for cellulase production. The enzyme produced by *Marinobacter* sp. (MSI032) was alkalotolerant, active at pH 9.0 [43]. Similarly, alkalotolerant cellulase from *Nocardiopsis* sp. SES28, isolated from Argentina was active at pH 8.0 and 40 °C [47]. *Bacillus mycoides* S122C show cellulase activity at 50 °C and pH 7.0 [48]. *Geobacillus pallidus* show cellulase production at 60°C [49]. Thermophilic bacterium *Caldibacillus cellulovorans* show cellulase activity at 80 °C [42].

Table 2: List of aerobic, anaerobic and facultative anaerobic bacteria producing cellulase

Bacteria	Growth condition	References
Balneomonas flocculans	Aerobic	[50]
Paenibacillus terrae ME27-1	Aerobic	[51]
Pseudomonas fluorescens	Aerobic	[52]
Paenibacillus cellulositrophicus	Facultative anaerobe	[53]
Cellulomonas uda	Facultative anaerobe	[54]
Halocella cellulolytica	Facultative anaerobe	[55]
Bacteroides cellulosilyticus	Anaerobe	[56]
Ruminococcus champanellensis	Anaerobe	[57]
Herbivorax saccincola	Anaerobic	[58]
Herbinix hemicellulosilytica	Anaerobic	[59]
Streptomyces reticuli	Aerobic	[60]
Streptomyces drozdowiczii	Aerobic	[61]

Table 3: List of novel bacterial strains producing cellulase.

Bacteria	Isolated from	References
Herbinix hemicellulosilytica	Biogas reactor	[59]
Herbivorax saccincola	Biogas reactor	[58]
Cohnella cellulosilytica	Buffalo faeces	[62]
Cellulomonas terrae	Soil	[63]
Cellulomonas composti	Cattle farm	[64]
Vibrio xiamenensis	Mangrove soil	[33]
Pseudomonas coleopterorum	Bark beetle	[65]
Bacteroides cellulosilyticus	Human gut	[56]
Ruminococcus champanellensis	Human gut	[57]
Paenibacillus cellulositrophicus	Soil	[53]

2.2.2. Fungal cellulase

Fungal cellulase have advantages over bacterial cellulase in having high yield and able to produce a complete cellulase system [66]. Cellulase producing fungi are ubiquitous and found in a wide variety of environments such as soil, decaying logs of wood, sawdust [67], forest soil [68], litter soil [69], mushroom compost [70], marine sample [71], bovine rumen [72], and even as endophytes [73]. Unlike bacterial cellulase, fungal cellulases were active over a wide range of temperatures. Psychrotolerant fungus, *Aspergillus terreus* AKM-F3 produce optimum cellulase at 15 °C [74], *Aspergillus niger* produce optimum cellulase at 30 °C [75]. *Nectria catalinensi*s cellulase activity ranged from 50 to 55 °C [76]. *Aspergillus fumigatus* M.7.1 and *Myceliophthora thermophila* M.7.7 produce cellulase at 70 °C [77].

Fungal cellulases are also active over a wide range of pH. *Penicillium citrinum* was reported for alkali stable cellulase [78]. Acid tolerant cellulase was reported by *Trichoderma reesei* [79]. Fungus with dual tolerance such as acido-thermo-tolerant cellulase by *Chaetomium thermophile* (pH 4.0-4.5 and 60°C) [80] and *Penicillium* sp. CR-316 and *Penicillium* sp. CR-313 (65 °C and pH 4.5) [68] was also reported.

3. Xylanase

Xylanases (EC 3.2.1.x) are glycosidases which catalyze the endohydrolysis of 1,4- β-D-xylosidic linkages in xylan. They are produced by a plethora of organisms including bacteria, algae, fungi, protozoa, gastropods, and anthropods [81].

There is a phenomenal increase in the use of microbial xylanase as they offer advantages over conventional chemical catalysts, including high catalytic activity, high degree of substrate specificity, high productivity, easily biodegradable, pose no threat to the environment, and are economically viable [82].

3.1. Enzymatic hydrolysis of xylan

Due to the complex structure of xylan, its hydrolysis includes different types of enzymes. Endo-xylanases are the enzymes that cleave the glycosidic bonds in the xylan backbone, bringing about reduction in the degree of polymerization of the substrate [83]. β-xylosidase (E.C.3.2.1.37) hydrolyze short xylooligomers into single xylose units. There is a significant variation in their mode of action of β-xylosidases when compared to exo-xylanases. Exo-xylanases act on the xylan backbone from the reducing end (exo-fashion) producing short-chain oligomers whereas β-xylosidases hydrolyze short xylooligomers into single xylose units [84,85,86]. α-D-Glucuronidases (E.C.3.2.1.139) cleaves the α-1,2-glycosidic bond of the 4-O-methyl-D-glucuronic acid side chain of xylan [87,86]. α-Arabinofuranosidases (EC 3.2.1.55) are enzymes known to release terminal α -1,2-, α-1,3- and α-1,5 α-L-arabinofuranosyl residues

from hemicellulose such as arabinoxylan and other L-arabinose containing polysaccharide [83]. Acetylxylan esterase (EC 3.1.1.72) acts on carboxylic ester bonds. Ferulic acid esterase and p-coumaric acid esterase cleave ester bonds on xylan [88]. Ferulic acid esterase cleaves the linkage between arabinose and ferulic acid side groups, while p-coumaric acid esterase cleaves between arabinose and p-coumaric acid [89].

3.2. Microbial source for xylanase

Horikoshi and Atsukawa in 1973 [90] reported the commercial application of xylanase obtained from alkaliphilic bacteria. However, studies on microbial xylanase started during the 1960s, but the main focus of the study was plant pathogen relation. The study suggests that, xylanase along with other enzyme degrade the plant cell wall which cause the infection [91]. Due to the wide application of xylanase, several fungi and bacteria were explored for the ability to produce xylanase.

3.2.1. Bacterial xylanase

Bacteria producing xylanase have been isolated from various environments such as soil sample [90], marine sample [92], hot-spring water [93], mushroom compost [94], poultry compost [95], human gut [96], and sheep dung [97]. Prevalence of the xylanolytic bacteria have been reported from most of the bacterial groups (Table 4).

Bacterial xylanases has been reported to be active at wide temperature range. *Pseudoalteromonas haloplanktis* TAH3A (XPH) and *Flavobacterium* sp. MSY-2 (rXFH) have been reported for producing psychrophilic xylanases [98]. *Kluyvera* sp. strain OM3 can produce high level of cellulase free xylanase at 70 °C [99]. Bacteria like *Thermonosporafusca*, *Bacillus stearothermophilus*, and *Dictyoglomus thermophilum* show an optimum xylanase activity at temperature ranging from 65 to 85 ºC [100,101,102]. The first report on xylanase from alkaliphilic bacteria, *Bacillus* sp. TAR-1 was reported by Horikoshi and Atsukawa (1973) [90]. Apart from psychrophilic thermophilic and alkalophilic, haloalkaline xylanase [92)] and acidic xylanase [103] were also reported.

Table 4: List of xylanase producing bacteria

Bacteria	References
Caldicoprobacter algeriensis	[104]
Pseudoalteromonas haloplanktis	[105]
Staphylococcus sp. SG-13	[106]
Bacillus circulans	[107]
Streptomyces actuosus A-151	[108]
Streptomyces matensis	[109]
Streptomyces sp. 7b	[110]
Bacillus licheniformis SVD1	[111]
Geobacillus thermodenitrificans	[112]
Pseudomonas sp.WLUN024	[113]
Nonomuraea flexuosa	[114]
Thermoanaerobacterium saccharolyticum NTOU1	[115]
Gracilibacillus sp. TSCPVG	[116]
Acinetobacter junii F6-02	[117]
Jonesia denitrificans	[118]
Dictyoglomus thermophilum	[102]

3.2.2. Fungal xylanase

Fungi are often selected for the production of xylanase, as their yield are much higher than bacteria and in addition they also produce several auxiliary enzymes required for the degradation of substituted xylan [83]. Unlike bacterial xylanase, fungal xylanases are active at high temperature, low temperature, acidic pH, alkaline pH and even salt tolerant [119,120,121]. *Trichoderma reesei* produces xylanase in the mesophilic range [121] while *Cladosporium* sp. produces cold-active xylanase [122]. The most thermostable xylanases that have been described so far are those derived from *Thermotoga* sp. FjSS3-B.1, *Thermotoga maritima, Thermotoga neapolitana,* and *Thermotoga thermaru*m which are active at temperatures ranging from 80 °C to 105 °C [123]. *Neocallimastix frontalis* produces xylanase active at acidic pH [124]. *Gloeophyllum trabeum* produces xylanase at high temperature (70 °C) under broad pH range (4-7) [125].

4. Application of Cellulase and Xylanase

4.1. Cellulase and xylanase in the textile industry

A lot of limitations have been imposed on the textile industry due to rising environmental pollutions caused due to chemicals used during the process. In order to combat this situation, enzymatic treatment has emerged as an eco-friendly solution [14]. Cellulase and xylanase are one among the enzymes which are extensively used in the textile industries [126,83]. In textile industry, cellulase and xylanase have been used in various steps such as, during fabric

softening [83], bio-stoning of denim garments [14,126], bioscouring [14], releasing the extra dye [126], improving textile brightness [127], and bio-bleaching [128]. The use of microbial cellulase has several advantages over traditional stone washing (which mainly include pumice stones) including high productivity, less work-intensive, safer environment, short treatment times and less wear and tear of machines [129]. Cellulase from *Penicillium occitanis* [130] and *Trichoderma reesei* found to be very efficient candidates for biostoning application [131]. Xylanase from *Bacillus pumilus, Bacillus stearothermophilus* SDX, and *Penicillium janthinellum* have been used in textile industries for de-sizing of cotton and micropoly fabrics. It also helps in lowering the wetting time of fabrics, bioscouring efficiency and reducing the weight loss of the fabrics [132, 133, 134]. Cellulase from *Trichoderma reesei* [135] and *Aspergillus niger* have been used for biopolishing of the fabric [136].

4.2. Cellulase and xylanase in paper industry

Cellulase and xylanase have been used in the paper industry to overcome the limitations of mechanical pulping processes such as refining and grinding [14,137]. Xylanase from *Streptomyces thermoviolaceus* [138], *Aspergillus sydowii* [139], *Trichoderma reesei* [121], *Bacillus pumilus*, *Aspergillus fumigates, Chaetomiumcellulolyticum, Thermomyceslanuginosus,* and *Aspergillus kawachii* have been used for bleaching [140, 83, 138].

Xylanase have been used to increase pulp brightness, removing metal cations, reducing the overall paper cost, reducing the beating time of pulp, and restoring of bonding [141]. Xylanase treatment can render the chlorine requirement [142]. Cellulase and xylanase were beneficial for deinking of different types of paper wastes. Xylanase from *Aspergillus niger* DX-23 [143] and cellulase from alkalotolerant *Fusarium* sp. [144] were suitable for deinking. Advantages of enzymatic deinking over chemical deinking includes, reduce alkali usage, improved fiber brightness, enhanced strength, higher pulp freeness, and reduced fine particles in the pulp [137].

4.3. Cellulase and xylanase in food industries

Cellulase and xylanase found applicable in many food industries. They have been used for juice clarification, improving the quality of bakery products, reducing the viscosity of nectars, alteration of fruits sensory properties, used for olive oil extraction, and during beer and wine production [137].

During the early 1930s, when fruit industries began to produce juice, macerating enzymes complex (cellulases, xylanases and pectinases) from food-grade microorganisms (*Aspergillus niger* and *Trichoderma* sp.) have been extensively used to increase the juice yield and to overcome the difficulties encountered during filtration [145].

In the baking industries, wheat (which consist hemicellulose) is the key material. The hemicellulose present in the wheat is water-insoluble and cause many problems [146]. Xylanase during baking helps to transforms water-insoluble hemicellulose into a soluble form, which helps to increase the volume and creating finer and more uniform crumbs [147]. Xylanase from *Aspergillus foetidus* showed a remarkable difference in water absorption [148]. Xylanase also improves the quality of dough in such a way that it does not stick to the machinery parts, making it more machine-friendly [147]. Xylanase also enhances the bread quality and extend the shelf life by reducing the staling rate [149].

Cellulase and xylanase plays an important role during beer and wine production [150, 151]. The first microbial enzyme used in the wine industry was a commercial pectinase from *Aspergillus.* However, in the early 1980s, it was suggested that *Trichoderma* β-glucanase could be successfully used for wine making from grapes which were infected with *Botrytis cinerea. Botrytis cinerea* deteriorate ripe grapes and produces a high molecular mass soluble β-(1,3) glucan with short side chains linked through β-(1,6) glycosidic bonds, which cause severe problem during wine filtration. *Trichoderma* β-glucanase hydrolyzes glucans that cause adverse effects during filtration of wine [145]. Endoglucanase II and exoglucanase II from *Trichoderma* showed maximum reduction in the degree of polymerization and wort viscosity [152].

In past few years, β-glucosidase has attracted considerable attention in the wine industry because of its ability to improve the aroma of wines by modifying naturally present, glycosylated precursors [153]. Apart from glycosidase, α-L arabinofuranosidase and β-D-glucopyranosidase also help to increase the aroma of wine [151].

4.4. Cellulase and xylanase in animal feed Industries

Cellulase and xylanase have been used in animal feed industries, which help to improve feed nutritional value, eliminate anti-nutritional factors present in the feed grains, degrade certain feed constituents to improve the nutritional value, and provide supplementary digestive enzymes [137]. Cellulase and xylanase as feed additive helps digestion in cows and increase milk production [154]. Xylanase from *Aspergillus niger* helps growth performance, nutrient digestibility, and non-starch polysaccharide degradation in broilers [155]. Wheat and barley based supplement with xylanase and β-glucanase showed significant increased body weight and feed efficiency of turkeys [156].

4.5. Cellulase and xylanase in treating agricultural and forest wastes

Forest and agriculture, accounts for highest lignocellulosic waste production [157]. The major components of lignocellulosic are biodegradable; however, these are unlikely to result in hazardous conditions when there is inadequate oxygen to assimilate the wastes. When

this occurs, there is considerable damage to economic activities and the environment as well [158]. Enzymatic hydrolysis offer perspectives degradation of such waste, because it is a more specific process and the products obtained are without the presence of undesirable products [159,160]. Cellulase can remove the apple pomace waste [161] while cellulase and xylanase together can degrade lignocelluloses waste (beech tree leaves) [162]. Cellulase and xylanase from *Aspergillus niger* F7 were efficient in degrading forest wastes such as *Toonaciliata, Celtrisaustralis, Cedrusdeodara* and *Pinusroxburghii* [163]. Exo-1,4-β-glucanase from *Trichoderma viride* has been efficiently used to remove orange peel waste.

5. Conclusion

Microbial cellulase and xylanase have shown their potential application in textile industries, paper industries, food industries, animal feed industries, juice industries, brewing industries, bioremediation and bio-refinery for forest and agriculture wastes. Due to immense industrial potential, microbial cellulases and xylanases represents potential candidates for research by both the academic and industrial research groups. Despite an increased knowledge of fungal and bacterial cellulases and xylanases, further studies to isolate potential cellulases and xylanases producing strains and direction for improving the process economics should be carried.

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7. References

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Advances in Biochemistry & Applications in Medicine

Chapter 5

Recent advancement to target Breast Cancer and Cancer Stem Cells

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1. Introduction

Breast cancer is the second most prominent reason of cancer death in women after lung cancer, and it accounts for 25.2% of all cancer in women [1]. In the US, approximately about one in eight women (12%) develop invasive breast cancer [2]. In 2015, approximately 40,290 US women were expected to die from breast cancer. There was an estimation of 231,840 new cases of invasive breast cancer among US women in 2015 [3]. The chance of breast cancer causes woman's death is about 3%. Various kinds of therapies such as hormonal, immuno-therapeutic agents, surgery and cytotoxic currently are being used to target the breast cancer. The response rate from these treatments comprises 60% to 80% for primary breast cancers and about 50% for metastases [4,5]. However, 20%-70% of patients showed reversion of cancer within five year of time [6]. Recurrence development allied with resistance to therapy and augmented death risk. In patients with primary breast cancer, combining cytotoxic and radiation therapy with anastrozole attained four-year survival rate of 91.6 % [7]. Gene mutations and dysregulation has been identified in breast cancers like the enhanced expression of the heparan sulfate interacting protein, p53 mutations (connecting with high histological grade) and mitochondrial D-loop mutation (allied with lymph node-positive breast carcinoma) [7].

2. Botanicals for Breast Cancer Prevention

In the 20th century, people throughout the world now are focusing on natural products to cure different diseases. Eventually it was recognized that some natural products could in some instances be made more effective through the use of chemistry to produce semi-synthetic compounds (i.e. aspirin and later the penicillins) [8,9]. *Vernonia amygdalina* is an eatable African mountain plant from the Asteraceae family. A study reported that *V. amygdalina* extract

has efficacy to inhibit the proliferative properties of MCF-7 and MKL-F cells [10,11]. Higher concentration of *V. amygdalina*, extracted by chloroform, hexane, butanol and ethyl acetate repressed the DNA synthesis by 76-98%. Flavonoids are the large class of pigments from the plant having a structure based on or alike to that of flavone. Most widely examined flavonoids are the catechins, flavopiridol, genistein, and quercetin. These flavonoids are known to inhibit the cancer proliferation and holds anti-tumour activities. Flavopiridol is a semisynthetic flavone derivative of the natural anti-inflammatory and immuno-modulatory alkaloid rohitukine [12]. For the first time, rohitukine was isolated from *Amoora rohituka* [13,14] and later from *Schumanniophyton magnificum* [15] and *Dysoxylum binectariferum* [16]. The study reported that rohitukine showed modest cytotoxicity against human HL-60 promyelocytic leukemia and HCT-116 colon cancer cells [17]. Green tea is a category of tea that is formed from *Camellia sinensis* leaves that have not undergone the same crushing and oxidation process used to make oolong and black tea. Green tea extracts exposed synergetic action with an anti-estrogens drug, tamoxifen. Tea extracts tested on estrogen receptor-positive MCF-7, ZR75, T47D human breast cancer cells in combination with tamoxifen were found that drug showed much more efficient by suppressing the cell proliferation than alone [18]. Another study reported that green tea inhibits angiogenesis. Crude green tea extracts and epigallocatechin gallate (main green tea polyphenol) reduced the transcription of vascular endothelial growth factor (VEGF) in a dose-dependent manner. *Withania somnifera* is a widespread shrub used in Asian traditional medicine. The organic extracts from *W. somnifera* were tested among two dozen selected plants [19,20]. Extract treated cell culture (murine fibrosarcoma cell line L929sA) shown sensitivity to TNF. The active ingredient in *W. somnifera* leaves and roots comprises C-28-steroidal lactone triterpenoids, with anolides were identified [21]. With aferin A inhibited the growth of breast cancer cell lines more efficiently than the standard. Resveratrol is a botanical polyphenol that provides numerous health benefits including cancer prevention. Different studies revealed the potential of resveratrol on cancer cell signaling pathways [22]. It has been established that the outcome of resveratrol administration in *in vitro* and *in vivo* experimental models depends upon the route of administration and dosage given [22]. In scientific community curcumin is a well-known phytochemical known for its anticancer potential. It is a phenolic compound derived from *Curcumin longa* (commonly known as turmeric). This phytochemical have tremendous ability to inhibit cancer cell cycle, induce apoptosis in cancer cells and check the metastasis ability of the cancer cells of different tissue origin [23]. Curcumin is known to target growth factors, transcription factors, cytokines and protein kinases in cancer cells. Literature depicts the diverse molecular targets modulated by curcumin in cancer cells and thereby providing prevention against the devastating disease [23].

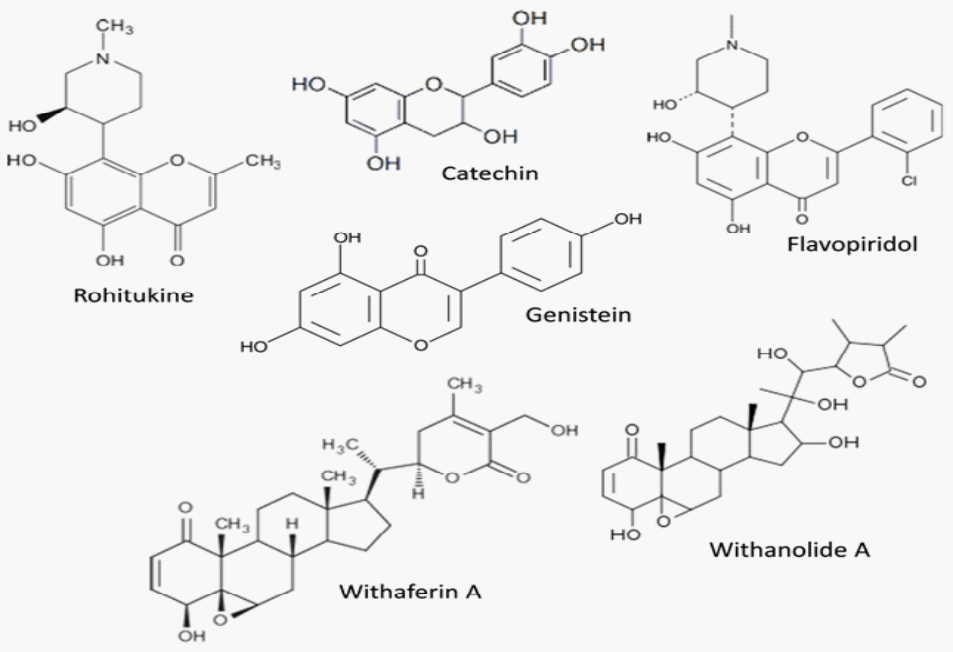

Figure 1: Structure of active natural phytochemicals

3. Multi-targeted Chemotherapy in Breast Cancer

Targeted therapy comprises the compounds which have potential to target the molecule responsible for cancer origin. However, conventional chemotherapy acts on the all dividing cells which have potential to generate toxic effect and normal tissues damage; targeted drugs allow hitting the target in a precise manner and cells subpopulations which have directly implicated in tumor progression. For the first time, targeted therapy starts with the trastuzumab (monoclonal antibody), acts against HER2 for the treatment of metastatic breast cancer and Imatinib, a small tyrosine kinase inhibitor targeting BCR-Abl, in chronic myeloid leukemia [24]. Multi-targeted therapy accommodates development of a multi-target drug which is likely to harvest a drug that has efficacy to cooperate with lower affinity than a single-target drug because smaller drug-like molecule will bind to an assortment of different targets with correspondingly high affinity.

Human epidermal growth factor receptor 2 (HER2) is also known as receptor tyrosine-protein kinase erbB-2, CD340 and neu. It comprises as a member of the human epidermal growth factor receptor family (EGFR/ERBB/HER). Over expression of the HER2 gene has been shown complicated in the development of the aggressive types of breast tumor. HER2 has become as an important biomarker in recent years. It is the target of therapy for around thirty percent of the breast tumor patients. Recently it has been reported that two HER2 targeted therapy showed more beneficial rather than one [25]. Comparable to women having the higher risk for recurrence, this therapy is not advantageous for women having the lower risk for recurrence.

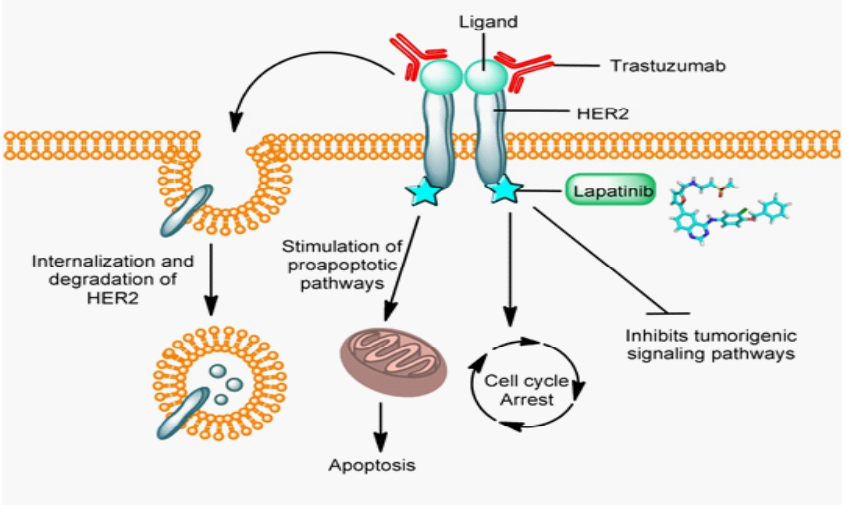

Figure 2: Current therapies for HER2-expressing breast cells. Constitutively active HER2 receptors dimerise with other HER receptors on the surface of HER2-expressing breast cancer cells, activating downstream signaling pathways that mediate tumorigenic cell proliferation, survival and invasion. Lapatinib binds to HER2 and HER1 and inhibits tumorigenic receptor signaling, promotes apoptosis and cell cycle arrest. Trastuzumab prevents constitutive activation of HER2, induces internalisation and degradation of the protein and stimulates the immune system to recognise HER2-overexpressing cells. Adopted and modified from [26].

Recently a study reported that benefit was modest in addition to second HER2 targeted medicine, pertuzumab (Perjeta) compared to standard of care trastuzumab (Herceptin) [25]. Women patient getting trastuzumab alone shown 93.2% of women were not able to develop invasive breast cancer while receiving trastuzumab and pertuzumab, 94.1% of women had not developed invasive breast cancer. Mechanism of action of trastuzumab targets only HER2 while pertuzumab targets both HER2 and HER3. Obstruction of cancer cell growth signals by antibodies against HER2 and HER3 enhances the lower chance of treatment resistance. Approximately 8% of all type of breast cancer patients have early HER2 positive breast cancer and may take advantage of these kinds of adjuvant therapy [25]. The study strengthens the concept of multi targeted drug therapy.

4. Identification of Novel miRNAs: New Paradigm in Breast Cancer Chemotherapy

In the multicellular organism, stem cells are the undifferentiated cell able to develop the same type of more cells and from several other kinds of cell arise by differentiation. From several decades, stem cells are using immuno-modulation or reconstitution, one of the methods employed for cancer treatment. Stem cells have self-renewal capability with extremely replicative potential in multi-lineage differentiation capacity [27]. Cancer stem cells are the cells present in the tumor having potential to initiate the tumor [28]. Healthy cells pertains the three principal characters:

1. Self-renewal potential

2. Strict controls on stem cell numbers

3. Capability of differentiation, divide and to produce all functional element of the particular

tissue. In comparison to healthy cells, cancer stem cells lost their control toward division and cell number. The cancer stem cells present in very minute amount in solid tumor growth. They are responsible for the development of tumor cells. Different markers have been identified and validated to isolate the breast cancer stem cells (Table 1).

Table 1: Surface markers used to isolate the breast cancer stem cells

Marker	Reference
$CD44^{+}CD49f^{hi}$ $CD133/2^{hi}$	[29]
ALDH1	[30]
$CD44^{+}/CD24^{-}$ CD133	[31]
CD49f	[32]
CD61	[33]

MicroRNAs are the small non-coding RNAs which have potential to control an extensive range of biological progressions comprising carcinogenesis. Various studies have been found that miRNAs are profoundly dysregulated in cancer cells. Under certain circumstances, miRNA may act as either tumor suppressor or oncogenic. miRNAs comprises 20 to 25 nucleotides in length binds to the 3' untranslated regions (UTR) of mRNAs leads to the repression of target proteins via mRNA degradation or translational inhibition.

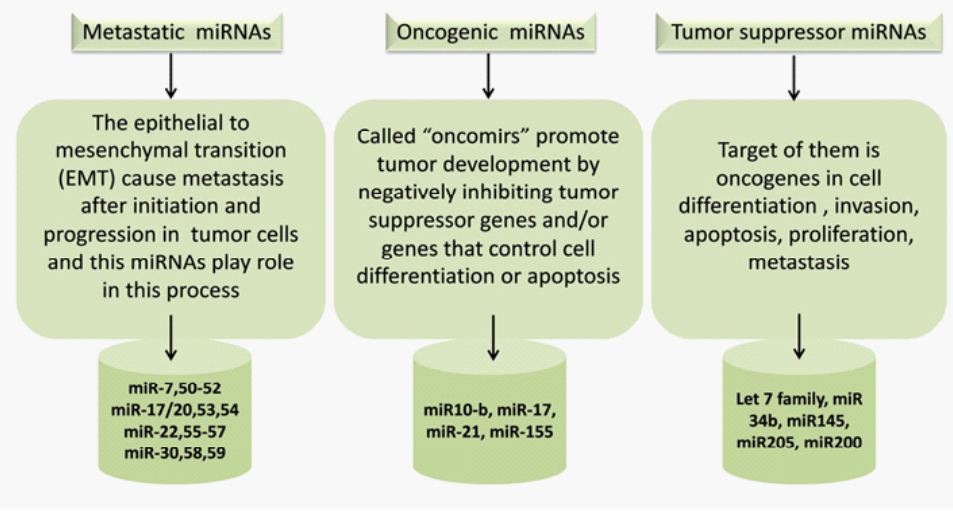

Figure 3: Classes of miRNAs in breast cancer. Adopted and modified from [34]

Transcription of the miRNA gene results in pri-miRNA formation in the nucleus which is cleaved by Drosha and DiGeorge Syndrome Critical Region Gene (DGCR8) proteins resulting in the generation of pre-miRNA transcript. The exportin-5 then exports the pre-miRNA from the nucleus to the cytoplasm where it gets processed by Dicer, and other enzymes such as trans-activator RNA (tar) - binding protein (TRBP) leading to the formation of short RNA duplexes. It then forms a miRISC/Ago complex with the help of some other proteins and factors. This complex then guides and performs some crucial functions in the cellular systems [35].

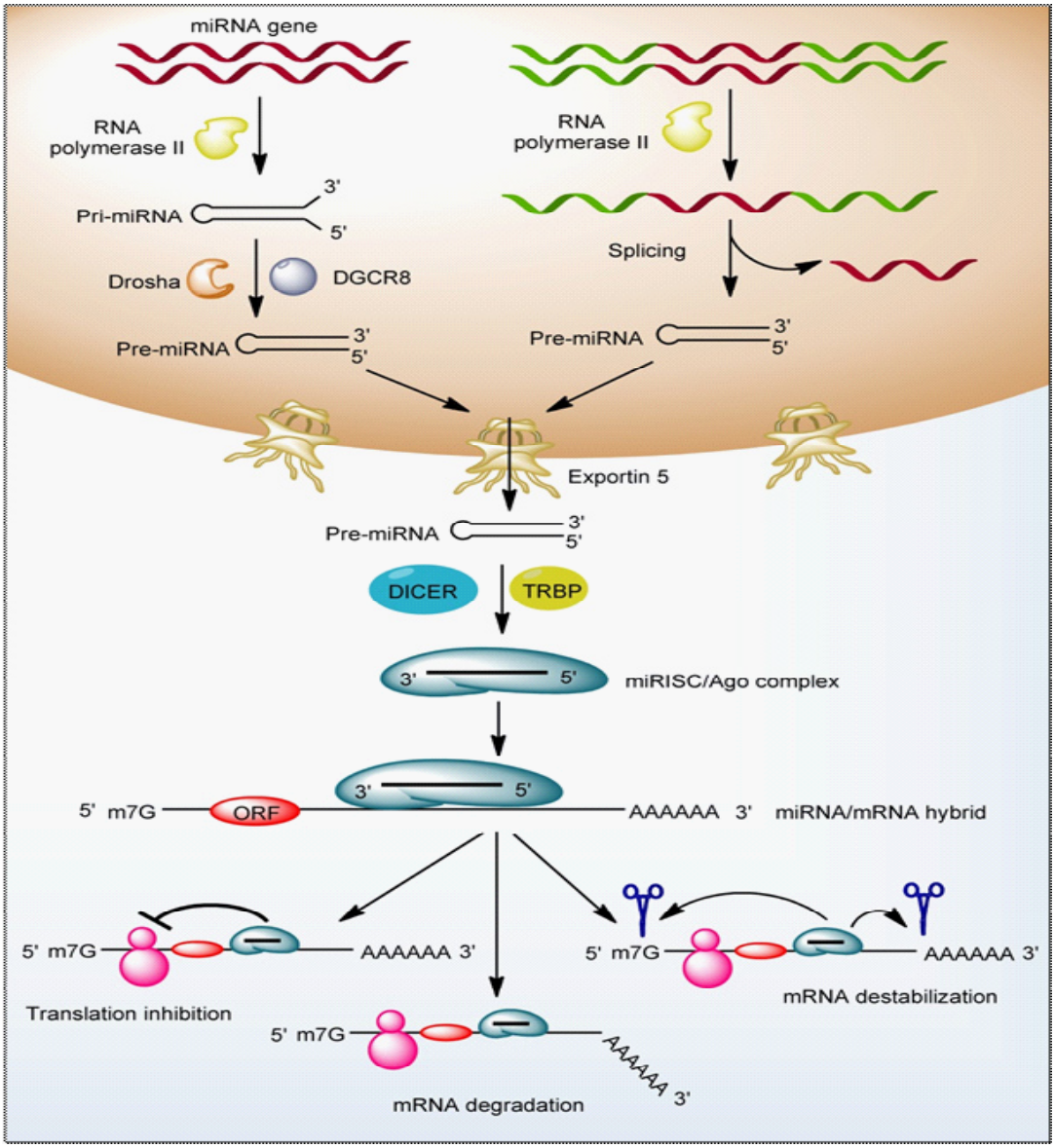

Figure 4: Biogenesis of miRNA and mRNA degradation. Adopted and modified from [35]

Recently a study reported that a small RNA molecule, miR199a, supports to sustain the activity of stem cells in both cancerous and healthy breast cells [36]. miR199a regulates various factors that assist noncancerous mammary gland stem cells (MaSCs) resist differentiation and retain their capacity to self-renew in human as well as mouse breast cancer stem cells. It supports MaSCs to hold their stem cell activity by repressing the production of a protein called ligand-dependent corepressor (LCOR), which binds DNA to control the gene expression. Up-regulated miR199a expression in mouse MaSCs reduced the LCOR proteins and improved healthy stem cell function. Conversely, increased LCOR expression diminishes mammary gland stem cell activity. Tumor-expressed higher expression of miR199a correlates with poor survival rates, while the tumor with enhanced levels of LCOR shown better prognosis [36].

LCOR sensitizes the cells against the effects of signaling molecules such as interferon released from immune (macrophages) and epithelial cells in the mammary gland. These cells secrete interferon alpha (IFN-α) to encourage the cell differentiation and cell division inhibition in the normal mammary gland development. The microRNA plays a crucial role during

tumorigenesis [37]. miR199a also protects MaSCs from IFN signaling and allowing them to remain undifferentiated and accomplished of self-renewal. Interferons have been extensively worked for the tumor treatment. These kinds of treatments become much more efficient if the cancer stem cells (interferon resistant) can be rendered sensitive by targeting the LCOR-mi-R199a pathway. These sorts of examinations expose a new property of breast cancer stem cells that give them compensations in their communications with the immune system. Therefore it characterizes an outstanding opportunity to adventure for refining cancer immunotherapy [36].

4. Combinatorial Immunotherapy: A New Hope in Early Stage Triple Negative Breast Cancer

Immunotherapy or biologic therapy is a technique for the treatment of cancer that boosts the body's natural defences to fight against cancer. This kind of therapy uses substances which are made by the body or in the laboratory to improve or to restore the immune system function. In the phase I trial, combining the immune checkpoint inhibitor (durvalumab/MEDI4736) with chemotherapy as a pre-operative treatment for early stage triple negative breast cancer (TNBC) revealed a 71% pathologic complete response to the combination treatment [38]. Chemotherapies solely generate a complete pathologic response at the rate of around 35-40% for women with TNBC. Combinational therapy is important because women with breast cancer who achieve complete response have excellent long-term survival. There is an essential requirement for new employment options to increase cure rates for women with TNBC. Phase I clinical trial sought to restrict the safety and tolerability of durvalumab in combination with weekly nabpaclitaxel and dose dense doxorubicin/cyclophosphamide (ddAC) [38]. There were no dose-limiting toxicities with the combination; Therefore the full dose of neo-adjuvant chemotherapy can be administered concurrently with the full dose of durvalumab. Among the seven patients in phase I clinical trial, five achieved the complete pathologic response, one had partial response with residual disease, and one had widespread residual cancer [38].

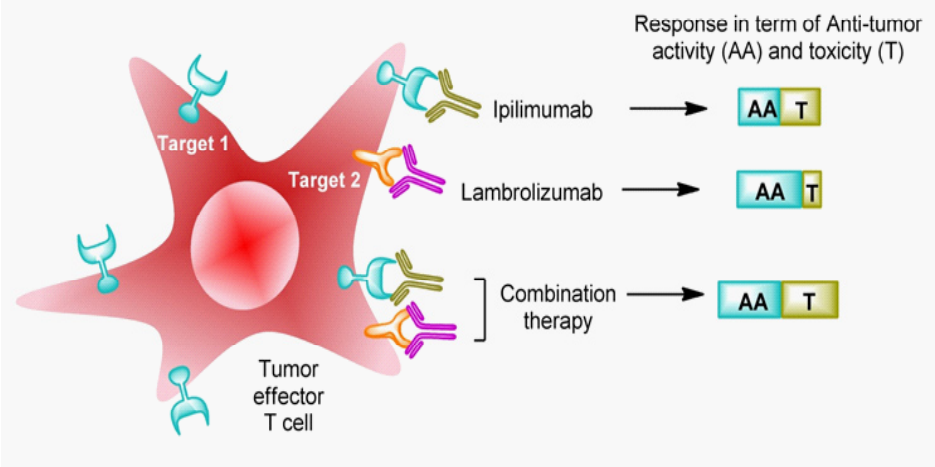

Figure 5: Targeting immune targets 1 and 2 by different inhibitor molecules, alone or in combination. Inhibition might results in differential levels of responses in terms of antitumor activity and toxicity. Blockage of target 1 by lipillimumab might results into anti-tumor activity and toxicity both in tumor cells. Blockage of target 2 by lambrolizumab might results into more anti-tumor activity and comparatively less toxicity in tumor cells. By using the concept of combinatorial therapy, blockage of target 1 and 2 by respective inhibitors might results into comparatively enhanced anti-tumor activity and toxicity in tumor cells. Adopted and modified from [39]

5. Novel PARP Inhibitor Against Advanced Breast Cancer

Enzyme Poly (ADP-ribose) polymerases (PARPs) have the capability to handover the ADP-ribose to the target proteins (poly ADPribosylation) [40,41]. Approximately 18 members of the PARP family have been reported yet, and several different genes encode these family proteins. PARP family shares homology in their conserved catalytic domain [40]. Several isoforms such as PARP1 and PARP2 are best known for their contribution to the DNA repair processes. Through the various studies, it is now clear that PARPs family have a necessary role in numerous cellular processes like proliferation and cell death [40].

In the majority, PARP substrates include nuclear proteins involved in nucleic acid metabolism, chromatin structure modulation, DNA synthesis and repair [41]. In the presence of DNA strand breaks, PARP also has potential to regulate and modify itself. It is the main acceptors of poly ADP-ribose *in vivo*. Best characterized PARP family member is the PARP1. PARP2 is narrowly associated to PARP1 with 69% resemblance in their catalytic domain and recognized by the perseverance of PARP activity in PARP1-deficient cells [40,42].

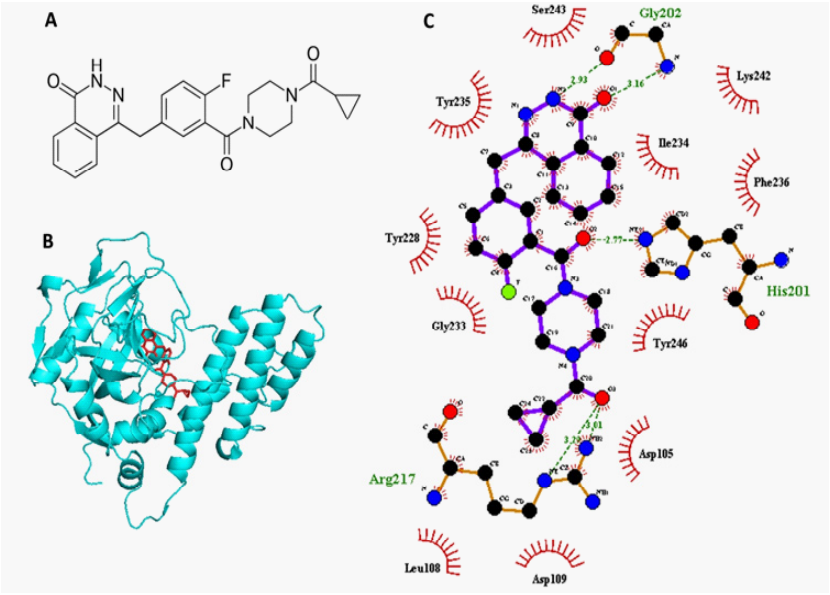

Figure 6 : (A) Structure of olaparib (PubChem CID: 23725625) (B) Cartoon structure of poly (ADP-ribose) polymerase (Cyan colour) (PDB ID: 3L3M) complexed with olaparib (Red colour). The 3D structure of the target receptor (PARP) was downloaded from RCSB-protein data bank in .pdb format. The olaparib structure was retrieved from NCBI PubChem compounds database in .sdf format. Offline docking tool such as Auto Dock Tools 1.5.6 (ADT) was used for to study the drug-protein interaction. PyMOL molecular visualization tool was used for visualization of the interaction pattern in the drug-protein complex.(C) Schematic representation of hydrogen bonds and hydrophobic interactions between olaparib and Poly (ADP-ribose) polymerase. Green colour represents hydrogen bonding (developed using Ligplot +v.1.4.5) (Ligand bond, Non-ligand residues involved in hydrophobic contact, Non-ligand bond, Corresponding atoms involved in hydrophobic contact, 2.39 Hydrogen bond and its length)

Lynparza (olaparib) significantly reduce the progression of breast cancer that is induced partially due to mutations in a gene called breast cancer (BRCA). BRCA gene mutations are responsible for around 3% of all breast cancers. A new study (phase III clinical trial) included 302 women with breast cancer that has spread to other organs. They were positive for BRCA gene mutation. Investigation of their breast cancer cells shown that cells have one of the hormone-sensing molecules either estrogen or progesterone or have none of these two hormone receptors along with HER2. These type of cancers termed as "triple negative breast cancers" [43].

Lynparza diminished the risk of cancer development by as much as 42% compared to additional conventional chemotherapy. Lynparza also showed much fewer side effects that conventional chemotherapy. In 60% patients receiving Lynparza, the tumor showed evident shrinkage. A similar reduction was noted in 29% women on standard chemotherapy. Serious side effects were seen in 37% women on Lynparza compared to 50% in women with conventional chemotherapy [43]. Lynparza (olaparib) inhibits the enzyme called poly ADP-ribose polymerase (PARP). Poly (ADP-ribose) polymerase (PARP) family proteins included some cellular processes such as DNA repair, genomic stability, and programmed cell death. BRCA gene works by stopping the repair of the damage caused by cancer to the DNA. PARP further prevents the damage repair by the cells. Lynparza can work only on cancers where the BRCA gene mutation is responsible for those diseases. Lynparza is already in the market for ovarian cancer that is caused by BRCA. Two other similar drugs for BRCA mutation induced ovarian cancer, include Zejula and Rubraca manufactured and made by Tesaro and Clovis Oncology respectively [43].

6. Targeting MUC1 Protein: A New Step Towards Targeted Drug Therapy in Breast Cancer

One of the O-linked glycosylated proteins called mucins plays an important defence function by forming a protective mucous barrier onto the epithelial cell surface. Mucins also take part and perform the crucial function in intracellular signaling [44]. It exhibited onto the apical surface of epithelial cells that line the mucosal cell surfaces of multiple tissues such as pancreas, stomach, lung, and breast providing lubrication and protection. When cells become malignant, the MUC1 protein changes and signals tumor cells to multiply. In a study, as cancer attacked formerly healthy mice, their immune systems provide antibodies to fight against the cancer cells [45]. Cancer cells illuminated green fluorescence, purporting the binding of the antibody to the abnormal MUC1 proteins and neglecting the healthy cells. Scientist signifies these kinds of new therapies as a "smart bombs" that can combat cancer cells by killing them without hitting any of the neighbouring healthy cells. That compares with traditional chemotherapy that kills whatever it touches, including healthy tissue [45].

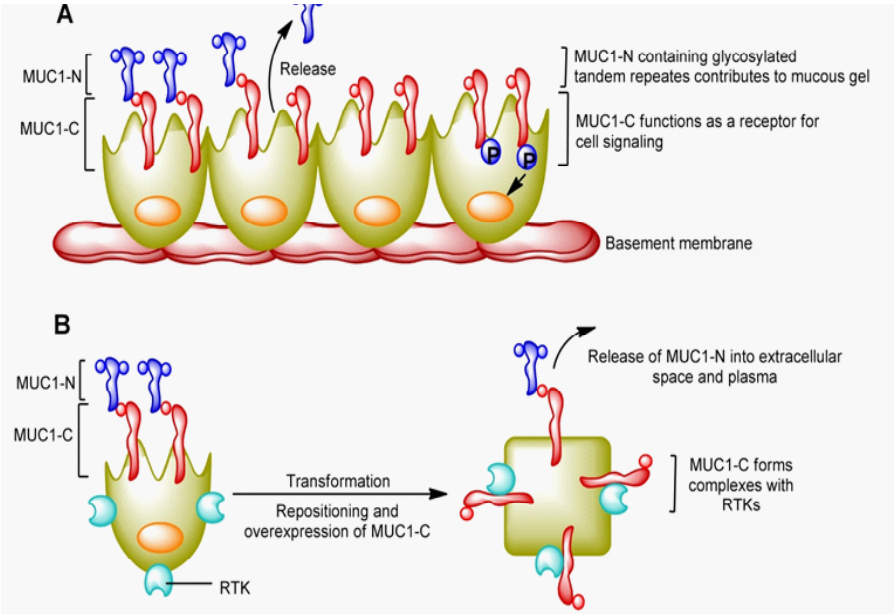

Figure 7: Aberrant localization and overexpression of MUC1-C in breast cancer cells. (A) The MUC1-N/MUC1-C heterodimer is expressed at the apical border of normal mammary epithelial cells. MUC1-N extends beyond the glycocalyx of the epithelial cells and contributes to the protective mucous barrier. With activation of the epithelial stress response and release of MUC1-N from the cell surface into the mucous gel, MUC1-C functions as a second line of defense to signal stress to the interior of the cell and thereby protects against loss of integrity of the epithelial layer. Figure modified from [46].(B) In normal epithelial cells, apical MUC1-C is sequestered from RTKs that localize to the basal–lateral borders. However, with transformation and loss of polarity, MUC1-C is repositioned over the entire cell membrane and forms complexes with RTKs that promote RTK signaling and up-regulation of MUC1-C expression. Figure modified from [46].

7. Sleeper Cells as Target for Breast Cancer Recurrence

Researchers have made tremendous advancement in saving the lives of patients diagnosed with breast cancer; the disease still comes reverse back after treatment in thirty percent of women. Treatments target the primary breast cancer, the seed cells called disseminated tumor cells (DTCs) have the potency to jump from the breast and resides in different parts of the body. Its restoration after a long time (some years) following as unusually aggressive type of cancers that are remarkably resistant to the therapy. It does not know that which patients are at risk of recurrence and the caretaker with no choice to take action against them but to watch and wait, often causing tremendous distress for patients. New research is providing clues that how these sleeper cells influence and new trials are giving hope to at-risk patients. Two clinical trials namely CLEVER and SURMOUNT are executing this important message to target the mechanisms that enable the cells to stay alive, as well as those that permit them to reactivate. Co-operators (women) in SURMOUNT trial who have taken breast cancer treatment are now in the watchful and waiting phase. Researchers are now capable of recognizing the patients with sleeper cells via a bone marrow test. Patients having positive outcomes, i.e., the appearance of DTCs can then registered in the CLEVER trial and assigned participants for the medications that will actively examine and kill the dormant cells before they can move towards the distinct parts of the body. These kinds of way can reconstruct the approach to care for patients having breast cancer and possibilities.

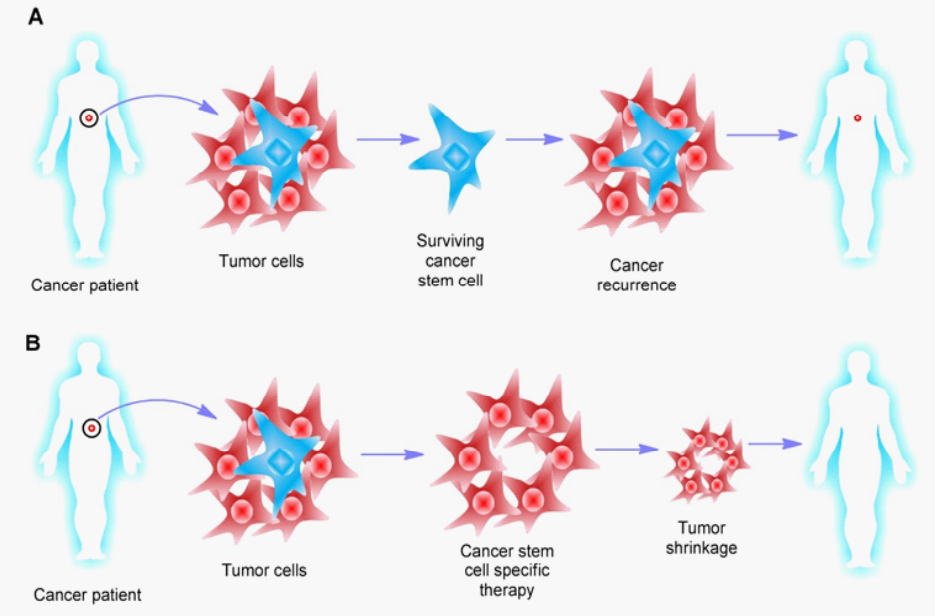

Figure 8: (A) Conventional approach to target the cancer remedies.Observation of the cancerous cells demonstrated that a special type of cell exists with the characteristics associated with stem cells called cancer stem cells. Cancer stem cells are tumor establishing and more prominently have the potency to give rise to all other cancer cell types found in a particular cancer. These stem cells of cancer can exist as a distinct population among other cells and cause metastasis and give rise to new tumors. Unfortunately, these cells can be very elusive and most of the cancer drugs have no or little effect on them. (B) In a novel approach, targeting stem cells can repress the chance of recurrence of cancer.

(Adopted and modified from https://ninithi.com/2015/07/26/smart-nanoparticles-to-target-and-destroy-cancer-recurring-stem-cells/)

8. Biological Production of Anticancer Secondary Metabolites

Ontario grown onions (Ruby Ring onion variety) contain one of the highest concentrations of quercetin, a type of flavonoid. The study revealed that the red onion has not only high levels of quercetin but also large amounts of anthocyanin which enriches the scavenging properties of quercetin molecules [47]. Anthocyanins are the water-soluble vacuolar pigments, provides colours to several vegetables and fruits. Depending on their pH, it produces different colours such as red, purple or blue. Anthocyanins pass sense that the red onions (darkest in colour) would have the most cancer-fighting power. Onions activate pathways that encourage cancer cells to undergo cell death. They develop an abnormal environmental condition for cancer cells, and they obstruct interaction between cancer cells which hinders growth. A new extraction procedure needs to be explored which will reduce the use of chemicals making the quercetin found in onions more suitable for consumption. Another extraction technique utilizes solvents that can transmit a toxic residue which is then ingested in food. Proper extraction strategy is must which can reduce the chemical residues is crucial to explore the onion's cancer-fighting properties in nutraceuticals as well as in pill form [47].

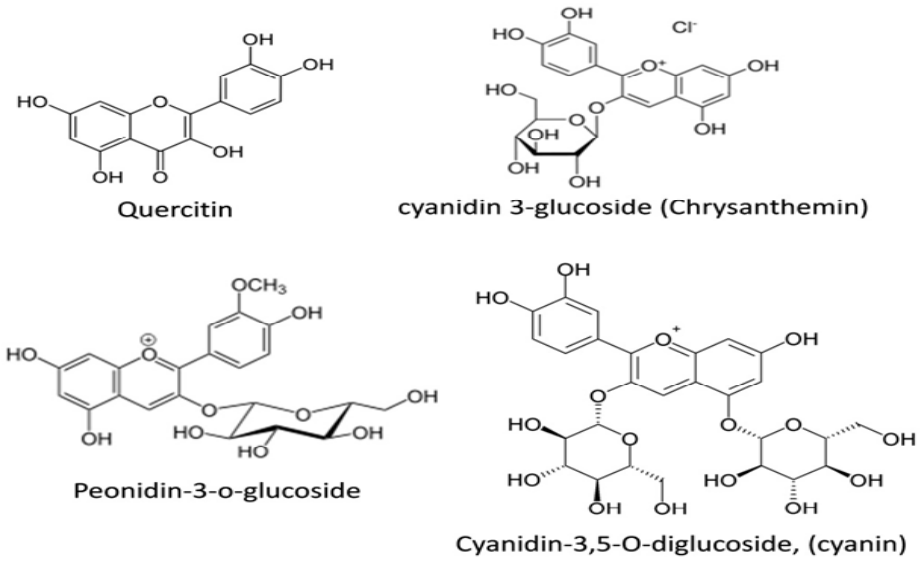

Figure 9: Quercitin and anthocyanins present in onion

9. Novel Biomarker for Identification of Early Stage Breast Cancer

The definition of the biomarker conferring to the National Cancer Institute is "a biological molecule found in body fluids, blood and other tissues that are a symbol of a normal or abnormal progression or disease such as cancer. Biomarkers characteristically distinguish an affected or diseased person from people without any disease. Variations can be due to some factors such as transcriptional alterations, somatic or germ line mutations and post-translational modifications. In the cellular systems, there is the astonishing variety of biomarkers is present such as proteins (enzymes or receptors), nucleic acids (microRNA or other non-coding RNA), antibodies and peptides [48]. A biomarker is a collection of various alterations of gene expression, metabolomic as well as proteomic signatures. It can be immediately identified in the circulatory system (plasma, serum or whole blood) or excretory secretion system (urine, stool, sputum or nipple discharge), and thus it can be easily evaluated [48].

Conventional treatment is inadequate for many early stage tumors that have grown past the in situ stage and failed to stop their spread to separate sites in the body. Recently researchers have identified SMARCE1 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1), a gene overexpressed in the subset of early stage cancers that are likely to become aggressively invasive making it conceivable to distinguish poorly invasive tumors from those that will likely expand and metastasize [49]. The researchers found that fifty percent of the early stage cancers with high SMARCE1 expression will metastasize at some point in the ten to fifteen years after their first diagnosis. SMARCE1 drives invasion by regulating the expression of secreted proteases that degrade basement membrane, an ECM barrier surrounding all epithelial tissues. In functional studies, SMARCE1 promotes invasion of *in situ* cancers growing within primary human mammary tissues and required for metastasis *in vivo* [49]. Mechanistically, SMARCE1 drives invasion by forming an SWI/SNF-independent complex with the transcription factor ILF3. In patients diagnosed with early-stage cancers, the

SMARCE1 expression is a strong predictor of eventual relapse and metastasis. Collectively, these findings establish SMARCE1 as a key driver of invasive progression in early-stage tumors [49].

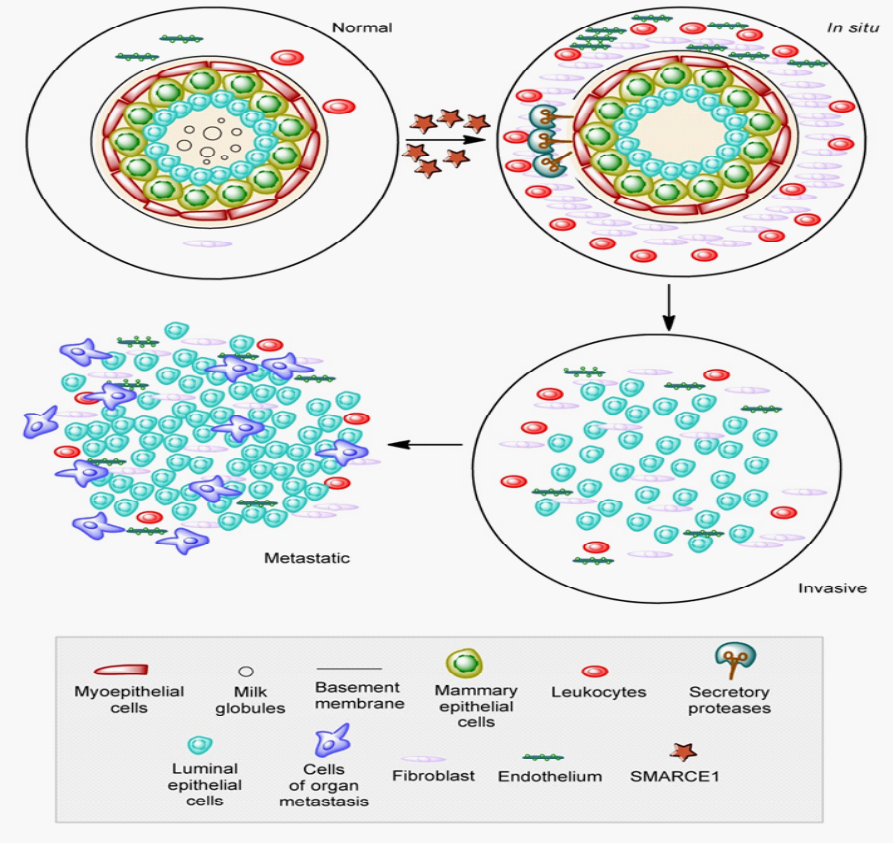

Figure 10: Schematic view of normal, in situ, invasive, and metastatic carcinoma progression. Normally, breast ducts consists basement membrane and luminal epithelial and myoepithelial cells. Stromaportion of the cells compriseseveral leukocytes, fibroblasts, and endothelial cells. Enhanced expression of SMARCE1 induces the protease expression which degrades the basement membrane. In in situ carcinomas the myoepithelial cells are altered by both phenotypically and epigenetically and their number decreases due to the basement membrane degradation. The number of stromal fibroblasts, lymphocytes, and endothelial cells increases with the same time. Myoepithelial cells and basement membrane loss results in invasive carcinomas, in which tumor cells can invade surrounding tissues andmight migrate to distant organs, ultimately leading to metastases. Adopted and modified from [50].

10. RIOKinase: A Novel Target for Breast Cancer

RIOK1 or RIO kinase 1 is one of the novel cytoplasmic component of the pre-40S pre-ribosomal particle(s) in mammalian systems, required for the final stages of cytoplasmic pre-40S maturation [51]. RioK1 is a new interactor protein of arginine methyltransferase 5 (PRMT5) which competes with pICln for interaction and modulates PRMT5 complex formation and substrate specificity [51]. RIOK1 may reveal new insights into defects underlying breast and related cancers and may reveal new therapeutic possibilities for these cancers. RIOK1 knockdown (not RIOK2 or RIOK3) in colon, breast and lung cancer cell lines exceedingly reduces cell proliferation and invasiveness [52]. These consequences mainly remarked in RAS mutant cancer cells. In contrast, the growth of RAS wild-type Caco-2 and Bcr-Abl-driven K562 cells is not influenced by the RIOK1 knockdown, recommending a precise obligation for RIOK1 in the context of oncogenic RAS signaling. Furthermore, RIOK1 activates NF-κE

signaling and promotes cell cycle progression. A study revealed that pro-invasive proteins metadherin and Stathmin1 to be regulated by RIOK1. In conclusion, RIOK1 could be a possible therapeutic target, particularly in RAS-driven carcinomas [52].

11. Summary

Over the past few decades, considerable progress has been made in breast cancer treatment. Beside that survival rates for patient of breast cancer remain poor which underlies the need for further advances in therapy. Effort of researchers and scientist worldwide towards the better efficacy of breast cancer therapy includes the recent advances in breast cancer treatment such as multi-targeted chemotherapy; identification of novel anticancer miRNAs; combinatorial immunotherapy; novel PARP inhibitors; identification of novel biomarkers for early breast cancer detection, biological production of anticancer secondary metabolites; and targeting O-linked glycoproteins, sleeper cells and RIOK1. Finally the advancement in breast cancer therapy and or drug discovery needs to be study in much more depth for further improvements in outcomes for patients with the disease.

12. Acknowledgment

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Advances in Biochemistry & Applications in Medicine

Chapter 6

Application of Hydrogen/Deuterium Exchange Mass Spectrometry in Structural Biology and Molecular Medicine

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1. Introduction

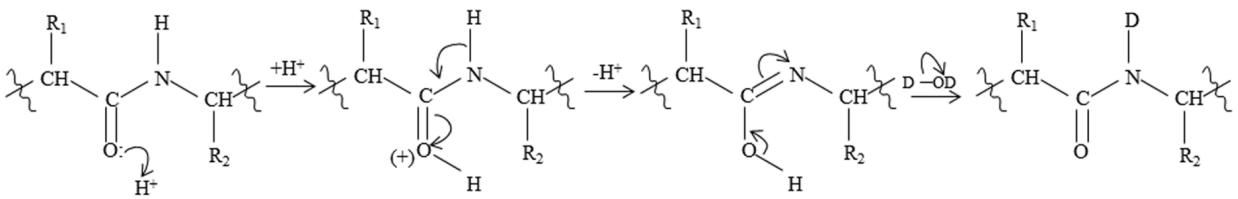
In last two decades soft ionization method in mass spectrometry has emerged as a powerful biophysical tool to study the structure of biological macromolecules such as proteins. Two ionization methods, electrospray ionization mass spectrometry (ESI-MS) and matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) were discovered independently by two research groups in 1980s. Although both of these methods have been extensively used in the analysis of primary structure of proteins in the domain of proteomics research, the isotope exchange chemistry of macromolecules monitored through soft ionization based MS made it feasible to study the functionally active three dimensional structure of proteins without any limit to its size, structural complexity and state of purity. The isotope exchange kinetics of polar hydrogen atoms of proteins with deuterium from solvent D_2O can be visualized using mass spectrometric platform. This isotope exchange kinetics monitored through MS is defined as hydrogen/deuterium exchange mass spectrometry (H/DX-MS). Subsequently the isotope exchange information can be used to investigate the change in conformational dynamics of a protein associated with a biological event such as protein-ligand interaction, protein folding etc. In addition, exploiting the permeability of D_2O across cell membrane, H/DX kinetics can be executed within a crowded molecular environment and the conformational dynamics of a

protein can be studied to understand the structure function correlation of a protein within a live cell.

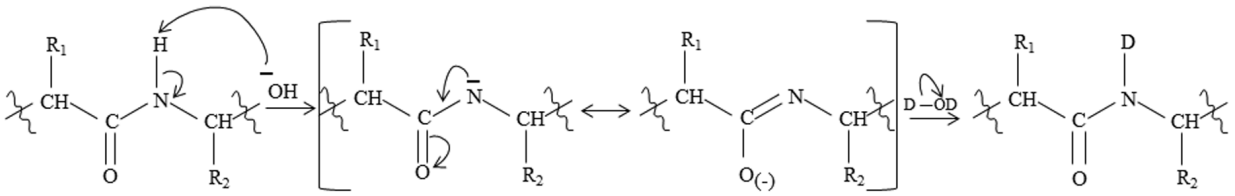
2. Fundamentals of H/DX Kinetics in Proteins

In solution phase, multiple vibrational degrees of freedom of a protein molecule provide flexibility in its conformation resulting in breathing motion of the molecule [1]. This conformational freedom results in local unfolding which causes transient open conformations to exist [2,3]. In general protein exists in a range of closely related conformations that are interconvertable at physiological temperature [4]. In a protein molecule hydrogen atoms bonded covalently to electronegative heteroatoms, e.g., oxygen, nitrogen and sulphur, are in continuous exchange with polar hydrogen atoms of the protonated solvent such as H_2O [5,6]. The rate of exchange depends on the differential solvent accessibility, inductive effect and field effect of neighbouring groups, pH and temperature [7,8]. Using mass spectrometry platform, the above exchange can be visualised by replacing the surrounding solvent water (H_2O) with heavy water (D_2O) [9-12]. In a protein molecule the polar hydrogen atoms present in the terminal groups and the side chain residues of amino acids exchange in the micro seconds scale and the kinetics of these fast isotope exchange steps cannot be monitored [13-15]. The time scale of the exchange reaction of the peptide backbone amide hydrogens is in the range of milli seconds, seconds, minutes and hours and thus it is possible to follow exchange kinetics in real time [16]. In general, the backbone amide hydrogens are involved in the formation of secondary structure of a protein, α helices and β sheets, and become less readily accessible in the exchange reaction to participate [17]. Eventually, the isotope exchange kinetics of backbone amide hydrogen can be translated to the conformational dynamics of a protein molecule [18,19]. The H/DX of the amide backbone can be catalyzed by both acids and bases [20,21]. Acid catalyzed mechanism initiates through the transient protonation of carbonyl oxygen atom followed by the removal of adjacent backbone amide hydrogen which gets replaced by the proton from solvent in the next step (**Scheme 1A**). In base catalysis, the hydroxide ion transiently abstracts the backbone amide hydrogen resulting in an imidate anion which gets subsequently protonated from the solvent (**Scheme 1B**).

Scheme 1A: Acid catalysis of hydrogen deuterium exchange



Scheme 1B: Base catalysis of hydrogen deuterium exchange



The rate constant of isotope exchange reaction kinetics k_{ex} can be expressed as follows:

$$k_{ex} = k_{H^+} x[H^+] + k_{HO^-} x[HO^-] + K_W \quad --------(1)$$

where, k_{H^+} and k_{H^-} are rate constants of acid and base catalyzed exchange reaction respectively and K_W is ion product of water. Differentiating both sides of equation (1) with respect to $[H^+]$ and applying the condition $dk_{ex}/d[H^+] = 0$, the H^+ ion concentration at which the exchange rate becomes minimum is as follows:

$$[H^+] = \left(\frac{k_{HO^-} x K_w}{k_{H^+}} \right)^{1/2} \quad -----(2)$$

For a typical backbone amide proton of proteins, k_{H^+} is 10^{-1} $M^{-1}s^{-1}$ and is 10^{7} $M^{-1}s^{-1}$ at 25 °C [22]. Therefore k_{ex} reaches its minimum value at pH 3. For every 10 °C rise in temperature the amide exchange rate is increased by about 2 to 3 folds [23].

3. Kinetics of Peptide Backbone Amide Hydrogen Deuterium Exchange

According to Linderstrom Lang's model, the local unfolding or breathing motion of protein molecules results in a number of transient open conformations, with exchangeable amide hydrogens being transiently exposed to the solvent. In the next step the amide hydrogen gets exchanged with its isotope from the solvent [24,25]. The isotope exchange reaction of amide hydrogens of protein can be represented as follows [26-28]:

$$NH_{closed} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} NH_{open} \xrightarrow[D_2O]{k_{ex}} ND \quad -----(3)$$

where, NH_{closed} and NH_{open} are the peptide backbone amide hydrogens in closed and open conformation respectively. ND is the isotopically exchanged amide NH. k_{op} and k_{cl} are rate constants of opening and closing events respectively and k_{ex} is the rate constant of intrinsic isotope exchange step.

The two states of the amide hydrogens NH_{closed} and NH_{open} can be collectively consid

ered as $NH_{backbone}$. In presence of large excess of solvent D_2O the rate law of $NH_{backbone}$ exchange can be expressed as:

$$\frac{d[NH_{backbone}]}{dt} = -k_{ex}[NH_{open}] \text{-----(4)}$$

Rate of change in concentration of intermediate NH_{open}

$$\frac{d[NH_{open}]}{dt} = -(k_{cl} + k_{ex})[NH_{open}] + k_{op}[NH_{closed}] \text{-----(5)}$$

Applying steady state approximation,

$$\frac{d[NH_{open}]}{dt} = 0 \text{-----(6)}$$

Thus, $(k_{cl} + k_{ex})\,[NH_{open}] = k_{op}\,[NH_{closed}]$

or, $(k_{cl} + k_{ex})\,[NH_{open}] = k_{op}\,([NH_{backbone}] - [NH_{open}])$

$$[NH_{open}] = \frac{k_{op}}{k_{cl} + k_{ex} + k_{op}}[NH_{backbone}] \text{ -----(7)}$$

Therefore, using equation 4 and 7

$$\frac{d[NH_{backbone}]}{dt} = -\frac{k_{ex}.k_{op}}{k_{cl} + k_{ex} + k_{op}}[NH_{backbone}] = -k_{HX}[NH_{backbone}] \text{ -----(8)}$$

where, k_{HX} is the experimentally measured H/DX rate constant,

$$k_{HX} = \frac{k_{op}.k_{ex}}{k_{cl} + k_{ex} + k_{op}} \text{ -----(9)}$$

Thus, in presence of large excess of D_2O the isotope exchange reaction follows pseudo first order kinetics,

$$\left[NH_{backbone}\right] = \left[NH_{backbone}\right]_0 .\exp\left(-k_{HX}.t\right) \text{-----(10)}$$

where $[NH_{backbone}]_0$ is the initial concentration of exchangeable backbone amide hydrogens.

Protein in its native state exists mainly in its closed conformation [29]. Thus it might be assumed that under physiological condition open conformation is unstable and transient [30]. Thus exchangeable peptide backbone amide hydrogens predominantly exist in the closed conformation. Therefore, $k_{cl} >> k_{op}$ and using equation 9, k_{HX} is given by,

$$k_{HX} = \frac{k_{op}.k_{ex}}{k_{cl} + k_{ex}} \text{ -----(11)}$$

The rates of H/DX are primarily determined by the rate of opening of the closed conformation or by the intrinsic isotope exchange rate. These two regimes are referred as EX_1 mechanism and EX_2 mechanism respectively. Under EX_1 condition $k_{ex} >> k_{cl}$, implying that H/DX occurs immediately upon first opening event [31-35]. Therefore, using equation 11, k_{HX} can be written as,

$$k_{HX} = k_{op} \text{ --------(12)}$$

In general, EX_1 mechanism is observed at high pH or in the presence of denaturants such as urea, guanidinium hydrochloride where global unfolding or denaturation of a protein molecule occurs [36-38]. Also at alkaline pH the intrinsic exchange rate (k_{ex}) is very fast [36]. Under EX_2 condition $k_{ex} << k_{cl}$, implying that several opening closing cycles occur before the amide labeling [39]. Hence using equation 11, k_{HX} can be expressed as,

$$k_{HX} = \frac{k_{op}.k_{ex}}{k_{cl}} \text{ -----(9)}$$

$$\text{or, } k_{HX} = K_{op}\, k_{ex} \text{ ----------(13)}$$

where $K_{op} = (k_{op}/k_{cl})$ is the equilibrium constant of opening event. Under physiological condition protein demonstrates the isotope exchange through EX_2 mechanism. At the experimental temperature and pressure, the free energy change associated with local fluctuation and opening of native structure is represented as:

$$\Delta G_{HX} = -RT \log_e K_{op} = -RT \log_e \left(k_{HX}/k_{ex}\right) = -RT \log_e \left(1/P\right) \text{ -----(14)}$$

where $P = k_{ex}/k_{HX}$ is protection factor providing information on the stability of native structure [40]. The backbone amide hydrogens that are located in the loop regions are unprotected and remain in the open conformation. H/DX in those regions occurs rapidly with rate constant equals to k_{ex}.

H/DX reaction can be performed in two modes: continuous labeling and pulsed labeling methods. In the present chapter, applications of continuous labeling method in the advanced biochemistry research and in the molecular medicine have been described. In continuous labeling method, the native protein molecule is incubated in a buffer solution prepared using D_2O as solvent and the isotope exchange kinetics is monitored as a function of time. As the isotope exchange step has its own reaction kinetics, the dynamics of a biological event of a protein at different states along with their intermediates can be monitored using H/DX kinetics for all those states separately. The H/DX kinetics of the backbone amide hydrogens across a specific region of the polypeptide chain of a protein reflects the conformational dynamics of that region of the protein in its respective state [41,42]. In the continuous exchange method the

isotope exchange reaction is quenched at different time points by reducing pH of the solution to 3 and temperature to 4 °C. Subsequently, the proteolytic digestion of exchanged protein is performed under quenched condition so that the back exchange of the incorporated deuterium to solvent H_2O is minimum. In general, pepsin is used as the proteolytic enzyme in the H/DX experiments of proteins.

4. Data Analysis

The number of deuterium incorporated 'D' in a proteolytic peptide at a given time 't' is calculated as follows [43] :

where M_t is the observed isotope average centroid mass of the deuterated peptide at time t, M_0 and M_∞ are the isotope average centroid mass of the undeuterated and fully deuterated peptides respectively and N is the total number of backbone amide hydrogens in a peptide excluding amino terminal hydrogens. As mentioned previously, at a fixed pH and temperature and in presence of large excess of D_2O, the H/DX of each amide hydrogen follows pseudo first order kinetics. Therefore, the number of amide hydrogen exchanged with deuterium at time t, ND of a peptide can be obtained as follows:

$$NH_{closed} + NH_{open} + ND = [NH_{closed}]_0 \quad \text{-----(16)}$$

or, $$[NH_{backbone}] + ND = [NH_{closed}]_0$$

As, $[NH_{backbone}]_0 = [NH_{closed}]_0$, equation 10 can be written as

$$[NH_{backbone}] = [NH_{closed}]_0 \cdot \exp(-k_{HX} \cdot t) \quad \text{-------(17)}$$

or, $$ND = [NH_{closed}]_0 \cdot (1 - e^{-k_{HX} \cdot t}) \quad \text{-------(18)}$$

Considering $[NH_{closed}]_0$ is equal to the total number of peptide backbone amide hydrogens 'N' (excluding amino terminal amide hydrogen) and replacing ND by D

$$D(t) = N - Ne^{-k_{HX} \cdot t} \quad \text{-----(19)}$$

In practice, every single backbone amide hydrogen has its characteristic isotope exchange rate constant.

Therefore, D (t) can be expressed as

$$\underset{i=1}{\overset{N}{D}}(t) = N - \sum \exp^{-k_i t} \quad \text{-----(20)}$$

where, k_i is the isotope exchange rate constant of i^{th} backbone amide hydrogen.

On the basis of magnitude of H/DX rate constants, backbone amide hydrogens of a peptide can be grouped into three categories as fast, intermediate and slow exchanging [44]. Thus, the expression of D(t) can be simplified as:

$$D(t) = N - \left(P_A e^{-k_1 .t} + P_B e^{-k_2 .t} + P_C e^{-k_3 .t}\right) \text{-----(21)}$$

where, P_A, P_B, P_C are the number of fast, intermediate and slow exchanging amide hydrogens and k_1, k_2, k_3 are average rate constants of the respective groups of amide hydrogens. The H/DX exchange rates and populations of different group of backbone amide hydrogens can be obtained by minimizing the sum of squared residuals (SSR) through performing multiple iterations steps using Microsoft Solver. The SSR for each data set can be calculated as given below:

$$SSR = \sum_i [(y_{i,obs} - y_{i,calc}) / y_{i,obs}]^2$$

$y_{i,obs}$ (observed data) and $y_{i,calc}$ (calculated data) can be calculated from equation 15 and 21 respectively. The combination of the kinetic parameters which gives the minimum SSR values is selected as the best-fit values of a given kinetic data set [45].

To understand the change in conformational dynamics of a protein associated with a biological event, a comparative analysis is required between six rate constants and six populations between initial and final states of the protein. In practice, it is very difficult to deal with twelve such kinetic parameters simultaneously. This complicated analysis step can be simplified by calculating the rate of isotope exchange reaction of different groups of amide hydrogens in both states of protein molecule by using the method of initial rate of a reaction [45]. The initial rate of pseudo first order H/DX reaction of a particular group of backbone amide hydrogens can be calculated from the product of rate constant (k_i) and the respective population of backbone amide hydrogens (P_i) of the group, which is the initial concentration of reactants in the isotope exchange reaction. It was hypothesized that the algebraic summation of the differential exchange rates across three groups of amide hydrogens between two states of a protein molecule mirrors the change in the conformational dynamics of the molecule associated with the biological event. A positive sign indicates increased flexibility and a negative sign indicates increased rigidity in the conformational dynamics associated with the state change of protein molecule.

5. Application of H/DX-MS in Structural Biology

5.1. Protein ligand interaction

In general most of the molecular interactions in a biological system are non-covalent in

nature such as protein-ligand interaction, where ligands can be small or large biomolecules including metal ions. These interactions are always driven by the binding affinity and the specificity of the ligand to the protein molecule. H/DX-MS is widely used for studying such interactions. This approach relies on the fact that binding of ligand to the protein restricts the solvent accessibility of the backbone amide hydrogens at the binding interface, thereby decreasing the H/DX rates of those backbone amide hydrogens. In addition, ligand binding to a protein might lead to the exposure or burial of backbone amide hydrogens in another part of protein molecule which modulates the conformational dynamics of the molecule resulting in an increase or decrease in the H/DX rates respectively. Thus, the comparison of these exchange rates of amide hydrogens between free and ligand bound states of the protein provides information on both the binding sites and the ligand induced conformational change of the protein molecule. Here we highlight few examples of the application of H/DX-MS in the structural characterization of protein-ligand interactions.

A recent study by Bennett and co-workers reported the unique binding site of laulimalide on microtubules using H/DX-MS. This study revealed that the binding site of laulimalide is located to the exterior of the microtubule on β-tubulin and also showed the structural perturbations of certain regions of proteins associated with the binding of laulimalide [46]. The ligand induced conformational change of human hemoglobin has been described from the difference in the H/DX kinetics of both oxygenated and deoxygenated states of hemoglobin [47,48]. Using H/DX-MS, Li *et al*. showed the binding region of the thermococcales inhibitor of the proliferating cell nuclear antigen (PCNA) designated as TIP, in PCNA-TIP complex [49].

Identification and characterization of an epitope in a specific antibody-antigen complex is a crucial step in developing therapeutic antibodies and vaccines. H/DX-MS is extensively used in the epitope mapping. Using H/DX-MS, Malito *et al.* mapped the binding of monoclonal antibody with the factor H binding protein (fHBp), the vaccine antigen of Neisseira meningitidis [50]. Similarly, Lu *et al*. reported that the open end of the β-barrel structure is the binding region of IL-1β to the Hu007, a humanized IgG1 monoclonal antibody [51]. Other examples include the epitope mapping of cytochrome C –E8, IL-13–CNTO607, and IL-17A–CAT-2200 [52].

5.2. Conformational stability of proteins

According to Anfinsen's thermodynamic hypothesis, the native structure of a protein is the one with lowest possible free energy in the physiological environment [53]. However, the native structure can undergo certain conformational changes attaining metastable states under some conditions in a living system [54]. Investigation of the stability of a protein provides important biological information. A decrease in the stability might indicate a tendency of a protein molecule to misfold and an increase in the stability might represent the bound state

with the ligand [55,56]. H/DX-MS has been immensely used to study the stability of proteins in solution phase. To illustrate this application, some of the examples are presented below.

Ghaemmaghami *et al.* developed a method, named as SUPREX (Stability of Unpurified Proteins from Rates of hydrogen deuterium Exchange) that involved the use of H/DX kinetics of an unpurified protein monitored through MS to study its stability. In this method, the protein was allowed to undergo H/DX at different concentration of the denaturant and the relative stability was measured. This study showed that the presence of maltose increased the stability of maltose binding protein in its native state [57]. Using H/DX-MS, Hodkinson and co-workers studied the conformational dynamics of β2-Microglobulin (β2m). This study showed that the amyloidogenic protein β2m on its dissociation from the major histocompatibility complex class 1 (MHC-1) becomes very unstable and exhibits a high conformational dynamics resulting in the formation of unfolding transitions with an increased tendency for aggregation [58]. The conformational dynamics of β2m measured by H/DX-MS showed that there was no significant correlation between the rate of EX_1 unfolding and fibril formation. Thus, authors concluded that existence of other fibril nucleation mechanisms besides global unfolding might have resulted in the formation of amyloid fibrils from β2m [59]. Two conformations of human growth hormone (hGH) at two different pH were investigated by Hamuro *et al*. The authors reported that in acidic pH hGH was more flexible and less stable when compared to its conformation in neutral pH [60]. In an another study McCammon *et al.* reported that the binding of the small molecule inhibitors to transthyretin would result in a substantial increase in the stability of the tetrameric protein molecule and thereby reducing its amyloidogenicity [61].

6. Application of H/DX-MS in Molecular Medicine

6.1. Biosimilar study

Monoclonal antibodies are emerging as potential drugs, therapeutic agents for various diseases [62]. A crucial step in the development of these biopharmaceuticals is the structural characterization of a particular protein. The functions of a protein is driven by its three dimensional structure. Thus, monitoring higher order three dimensional conformation of monoclonal antibodies become crucial in the development of biosimilars, the generic version of originator proteins as drugs. Alterations in the structure of biosimilars might cause significant changes in its function and eventually their use as therapeutics leading to side effects in the living system [63]. H/DX-MS platform has been used as a potential biophysical tool in the biosimilar studies. Using H/DX-MS, Nakazawa *et al*. analysed the stability of oligomers of the formulated insulin analogs with different pharmacokinetics. The study showed that the difference in the H/DX rates exist among insulin analogs and a correlation was observed between the pharmacokinetics parameters in humans and the H/DX kinetics for the formulated insulin analogs [64]. In an another study by Vissar *et al*. characterized the proposed biosimilar rituximab (GP2013)

and compared with the originator rituximab using H/DX-MS. The study showed no significant structural change on addition of drug conjugate to the antibody (IgG). Authors also showed that GP2013 was physiochemically and functionally similar to the originator rituximab [65]. Similar studies were performed in characterization of biosimilars of Trastuzumab which is a humanized immunoglobulin used in treating HER2-overexpressing breast cancer and Cetuximab, a chimeric mouse-human immunoglobulin monoclonal antibody that is used in treating colorectal cancer and squamous cell carcinoma of head and neck [66].

6.2. Discovery of drug target sites

In order to develop drugs that can arrest the process involved in the undesirable pathological condition, it is important to structurally characterize protein molecules to obtain the information about the molecular mechanism associated with the disease states. H/DX-MS is one of the molecular approaches that provide information on the probable drug target sites in the protein molecules [67]. For example, the main pathophysiology of sickle cell anemia involves energetically favoured polymerization of sickle hemoglobin in the deoxy state of hemoglobin. Using H/DX-MS, Das *et al*. showed that the change in the conformational dynamics of amino acid residues around hydrophobic patch, alteration in the conformation of the groove regions and the modulation of the important axial contacts within a single stranded polymer and the lateral interactions between the amino acid residues across the double strands in the oxy state of sickle hemoglobin molecule might be the molecular basis of inhibition of sickle hemoglobin polymerisation in its oxygenated state [68]. Authors proposed that these sites with altered conformation might be utilized to develop drugs that can inhibit hemoglobin polymerization in sickle cell anemia.

7. *In vivo* Application

Proteins are workhorse within a live biological cell. Function of a protein is defined by its three dimensional structure. Functionally active conformation of a protein is not static but dynamic. Structure-function correlation of a protein *in vivo* can be explored by studying the change in conformational dynamics of a protein molecule associated with the biological event in an extremely crowded molecular environment of a live cell. Exploiting the permeability of D_2O across the cell membrane, H/DX can be executed inside a live cell. Subsequently the structural information related to conformational dynamics and conformational stability of proteins can be extracted.

7.1. Conformational dynamics of a protein *in vivo*

Narayanan et. al. studied that the structural transition of human hemoglobin associated with its oxygenation inside live Red Blood Cells (RBCs) [45]. The cooperativity and allosteric regulation of oxygenation and the associated change in the conformational flexibility across

various regions of both α and β globin chains of human hemoglobin were monitored through H/DX-MS of fully oxygenated and fully deoxygenated RBCs in separate sets by incubating RBCs in 300 mOsM buffer prepared in D_2O, which is isosmotic to human blood. The different steps involved in the H/DX of live cells followed by mass spectrometric data analysis are shown in **Figure 1**.

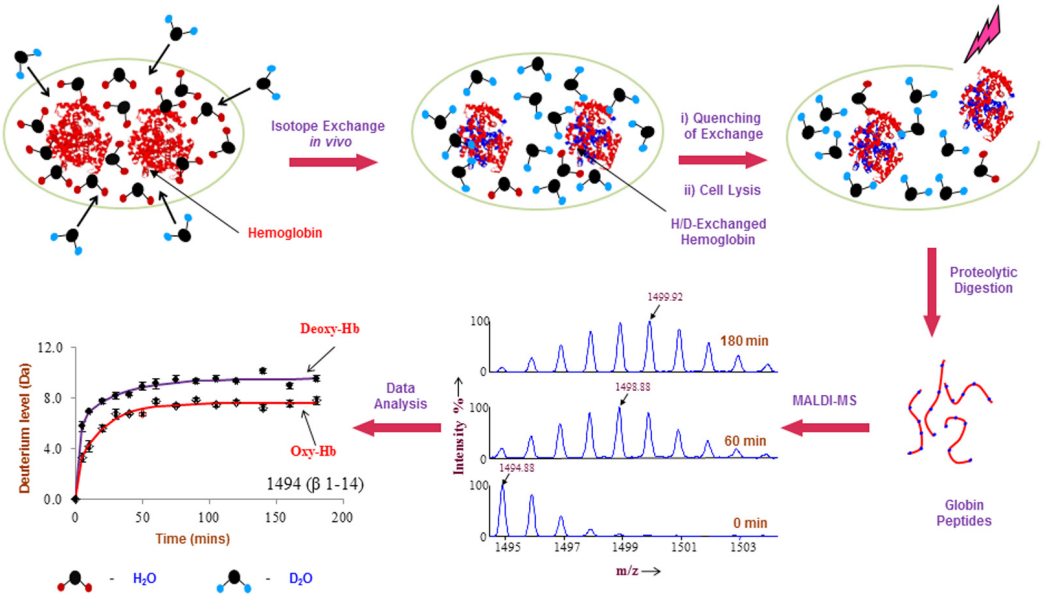


Figure 1: Schematic representation of hydrogen deuterium exchange in a living cell and subsequent steps involved in the analysis of exchange kinetics. ("Adapted with permission from (Anal Chem, 2015; 87: 11812-11818). Copyright (2015) American Chemical Society")

The rate of H/DX reaction of seven β globin peptides of human hemoglobin were calculated using the method of initial rates. The algebraic summation of the exchange rates of different parts of hemoglobin were used to understand the associated changes in the conformational dynamics of human hemoglobin inside live RBCs on deoxy to oxy transition. **Figure 2A** shows the changes in the conformational dynamics of β globin chain of hemoglobin associated with deoxy to oxy transition. The spatial orientation of those seven peptic peptides of β globin chain in human hemoglobin crystal structure is shown in **Figure 2B**. Hemoglobin concentration in RBCs is more than 95% of its total protein content. Thus the observed conformational change monitored through H/DX-MS was correlated with the literature information on the structural transition of human hemoglobin associated with its oxygenation *in vitro*. Authors proposed that this *in vivo* H/DX method might be successfully used to monitor conformational dynamics of any protein in cell irrespective of its size, location and structural complexity. For low abundant proteins in cell, liquid chromatography based pre-fractionation of isotope exchanged peptides at low temperature and acidic pH and subsequent online analysis of their mass might be helpful in the analysis.

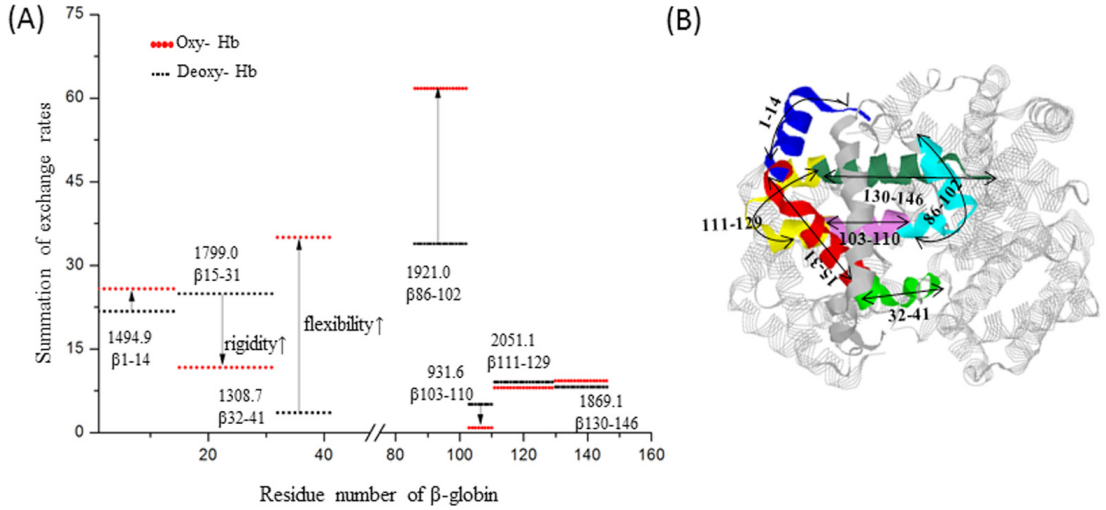


Figure 2: Conformational dynamics of β-globin chain of human hemoglobin on oxygen binding. (A) Hydrogen deuterium exchange rates of 7 different peptic peptides in both oxy and deoxy states of β-globin are shown. Direction of arrow indicates the increase in flexibility. ("Adapted with permission from (Anal Chem, 2015; 87: 11812-11818). Copyright (2015) American Chemical Society") (B) Orientation of 7 peptic peptides of β-globin chain is shown in the deoxy human hemoglobin crystal structure [PDB: 4HHB obtained from the protein data bank (www.PDB.org)].

7.2 Conformational stability of a protein *in vivo*

Ghaemmaghami *et al*., measured the thermodynamic stability of monomeric form of the N-terminal domain of repressor, $\lambda_{6\text{-}85}$, within the *E.Coli* cells using SUPREX [69]. Exploiting permeability of urea, a denaturant, and D_2O across the cell membrane, globally exchanging hydrogen exchange rate of the unfolded protein was measured and subsequently the thermodynamic stability of the protein in *E.Coli* cytoplasm was calculated. Record et al. reported that the presence of natural osmolyte at high concentration in the cytoplasm of *E.Coli* cell results in reduced water content, thereby increasing the stability of less hydrated native state [70,71]. In this study, *in vivo* measurement of H/DX was possible to monitor the stability of $\lambda_{6\text{-}85}$ till 3 M urea. Beyond 3 M urea cell viability dropped drastically, presumably due to irreversible denaturation of crucial proteins in cell.

According to the classical H/DX kinetics the observed rate constant of exchange for each hydrogen in terms of rate constants of various elementary steps of exchange reaction is given by equation 9,

$$k_{HX} = \frac{k_{op} . k_{ex}}{k_{cl} + k_{ex} + k_{op}}$$

Under EX_2 regime both k_{cl} and $k_{op} >> k_{ex}$. Thus,

$$k_{HX} = \frac{K_{op} . k_{ex}}{K_{op} + 1} \quad \text{-----(23)}$$

where, $K_{op} = (k_{op}/ k_{cl})$ is the equilibrium constant between closed and open conformations of protein. For hydrogen that is exchanging through global unfolding mechanism $K_{op} = (1/K_{fold})$,

$$k_{HX} = \frac{k_{ex}}{(1+K_{fold})} \quad \text{-----(24)}$$

For global unfolding the intrinsic isotope exchange rates (k_{ex}) is similar for entire peptide backbone amide hydrogens and the average H/DX rate of unprotected amide hydrogens is represented as $<k_{ex}>$. Thus the increase in mass as a function of time can be expressed as follows (7):

$$\Delta M = \Delta M_{\infty} + (\Delta M_0 - \Delta M_{\infty})\, e^{-k_{HX}.t} \quad \text{-----(25)}$$

where ΔM_0 and ΔM_{∞} are the increase in mass before global exchange and after complete exchange respectively [57]. Thus,

$$\Delta M = \Delta M_{\infty} + (\Delta M_0 - \Delta M_{\infty})\, e^{-[<k_{ex}>/(1+K_{fold})]t} \quad \text{-----(26)}$$

where $K_{fold} = e^{-(\Delta G_f + m[urea])/RT}$ -----(27)

ΔG_f is the free energy change on folding and m = (d[ΔGf] /d[urea]). Equation 26 was used to fit H/DX rates for both *in vivo* and *in vitro* experiments. From the measured stability parameter of protein it was concluded that in vivo stability of $\lambda_{6\text{-}85}$ in cell is very similar compared to its *in vitro* stability.

8. Conclusion

H/DX-MS can be used to monitor the change in the conformational dynamics of a protein associated with a biological event and to measure the conformational stability of a protein in solution phase. As the molecular mass is probed in an isotope exchange experiment, the method is applicable even in an extremely crowded molecular environment such as inside a live cell. Using this approach, structurally very complex protein molecules can be studied irrespective of its molecular size. The method has been increasingly used both in academic research as well as in molecular medicine such as in the biosimilar study. Advancement of liquid chromatographic separation of a complex mixture of proteolytic peptides with isotope exchange information retained, followed by online mass spectrometry based analysis might be an indispensable technique to understand innumerable cellular event at the molecular level in near future.

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