DOCKING AND MOLECULAR DYNAMICS SIMULATION STUDIES OF INSULIN-β-CYCLODEXTRIN INTERACTIONS

by

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LIST OF ABBREVIATIONS

μVT	constant chemical potential, volume and temperature
Ala	Alanine
APZ	Aripiprazole
Arg	Arginine
Asn	Asparagine
β-CD	beta cyclodextrin
Cys	Cysteine
DM	Diabetes Mellitus
DNA	deoxyribonucleic acid
DSSP	Database of Secondary Structure of Protein
ER	Endoplasmic Reticulum
FTIR	Fourier Transform Infrared
Glu	Glutamic acid
Gln	Glutamine
Gly	Glycine
GROMACS	Groningen Machine for Chemical Simulation
His	Histidine
HSA	human serum albumin
Ile	Isoleucine
LGA	Lamarckian Genetic Algorithm
MD	molecular dynamics
MSD	mean square displacement

Leu	Leucine
Lys	Lysine
MC	Monte Carlo
NMR	Nuclear Magnetic Resonance
NPH	constant number of molecules, pressure and enthalphy
NPT	constant number of molecules, pressure and temperature
NST	constant number of molecules, stress and temperature
NVE	constant number of molecules, volume and energy
NVT	constant number of molecules, volume and temperature
PBC	Periodic Boundary Condition
PDB	Protein Data Bank
PDB ID	identification of entries in Protein Data Bank
Phe	Phenylalanine
PMAA	Polymethacrylic Acid
PME	Particle Mesh Ewald
PM3	Parameterized model number 3
Pro	Proline
RAM	Random Access Memory
RNA	ribonucleic acid
RMSD	root mean square deviation
RMSF	root mean square fluctuation
R_g	radius of gyration
SASA	solvent accessible surface area

Ser	Serine
SPC	Simple Point Charge
Thr	Threonine
Tyr	Tyrosine
Val	Valine

LIST OF SYMBOLS

$\Delta G_{binding}$	binding free energy
ΔG_{conf}	free energy arises from the conformational changes in protein and ligand
ΔG_{int}	free energy of specific protein-ligand interactions
ΔG_{rot}	free energy loss caused by freezing of the internal rotations
$\Delta G_{solvent}$	free energy contribution with solvent effects
$\Delta G_{t/r}$	free energy changes in translational and rotational modes
ΔG_{vib}	free energy changes in vibrational modes
ΔS_{conf}	loss of conformational entropy upon binding to protein
ϵ	depth of potential well
σ	finite value of r
u_{LJ}	the Lennard-Jonnes potential
a_i	acceleration of molecule i
C_v	total heat capacity of system
F_i	force exerted on molecule i
m_i	mass of molecule i
ms	millisecond
nm	nanometer
nm^2	nanometer square
ns	nanosecond
R_c	center of mass of N atoms of insulin
r_i	vector of Cartesian coordinates of the i-th atom

r_i^{MD}	position of the atom i in the MD simulation
r_i^r	i position of the atom i in the reference structure
r_i^{ref}	reference position of particle i
$r^2(t)$	mean square displacement
T	actual temperature
T_τ	temperature time constant
T_0	desired temperature
U	potential energy of the system
V	velocities Verlett algorithm
v_i	velocities of molecule i

KAJIAN PENDOKKAN DAN SIMULASI DINAMIK MOLEKUL TERHADAP INTERAKSI INSULIN-β-SIKLODEKSTRIN

ABSTRAK

Interaksi protein-ligan memainkan peranan penting dalam menyediakan produk farmaseutikal yang baharu. Kajian ini merupakan usaha untuk memahami struktur dan dinamik kompleks insulin-siklodekstrin sebagai formula insulin oral yang baharu. Pendokkan dan simulasi dinamik molekul telah dijalankan untuk mengkaji interaksi antara monomer insulin dan dimer insulin terhadap β-siklodekstrins (β-CDs). Kajian pendokkan molekul berganda telah dijalankan menggunakan program Autodock v4.2 untuk menentukan bilangan β-CD yang boleh terikat pada tapak ikatan insulin selain menentukan konformasi insulin-β-CD yang paling stabil. Pendokkan molekul dengan 100 struktur rawak menggunakan konformasi awal nisbah monomer insulin kepada β-CD dan dimer insulin-β-CD 1:1 telah dijalankan dan daripada struktur pendokkan terakhir, β-CD telah ditambah dan proses diulangi sehingga peningkatan tenaga didapati. Keputusan pendokkan molekul menunjukkan maksimum empat molekul β-CD boleh terikat kepada struktur insulin dan nisbah insulin kepada β-CD 1:3 menghasilkan tenaga bebas pengikatan terendah. Selain pembentukan ikatan hidrogen, keputusan pendokkan menunjukkan bahawa interaksi hidrofobik memainkan peranan penting dalam menentukan kestabilan kompleks insulin-β-CD. Simulasi dinamik molekul 100 ns seterusnya dijalankan untuk membandingkan keputusan yang terhasil daripada kajian pendokkan molekul. Analisa daripada simulasi dinamik molekul mengesahkan kestabilan pada kompleks dimer insulin-β-CD 1:3 dengan nilai sisihan punca min

kuasa dua (RMSD) sebanyak 0.40 ± 0.02 nm selepas 38 ns menunjukkan perbezaan yang sangat besar berbanding sistem insulin bebas. Peningkatan nilai luas permukaan boleh dicapai pelarut (SASA) pada kompleks 1:3 insulin dimer-β-CD selaras dengan interaksi hidrofobik dalam sistem kompleks, oleh yang demikian menambahkan lipatan dan kestabilan insulin. Profil pergolakan punca min kuasa dua (RMSF) menunjukkan pengurangan fleksibiliti terhadap residu asid amino dalam insulin dimer dalam sistem kompleks berbanding sistem insulin bebas. Seterusnya, struktur dan dinamik asid amino pada setiap tapak ikatan dan kesannya terhadap β-CD turut dikaji. Keputusan menunjukkan β-CD terikat stabil pada setiap tapak ikatan pada insulin dimer dengan pergerakan terhad sekitar nilai minima. Selain itu, asid-asid amino berkutub seperti sistina (Cys62), serina (Ser63) dan glutamina (Gln66) terlibat dengan kekerapan tertinggi dalam pembentukkan ikatan hidrogen masing-masing dengan nilai peratusan 74.0 %, 54.3 % dan 51.9 %. Keputusan teori ini menunjukkan kewujudan interaksi penting antara insulin dan β-CD yang mana boleh menyumbangkan maklumat penting terhadap formulasi insulin.

DOCKING AND MOLECULAR DYNAMICS SIMULATION STUDIES OF INSULIN-β-CYCLODEXTRIN INTERACTIONS

ABSTRACT

Protein-ligand interactions play an essential role in the design of new pharmaceutical products. This study attempts to understand the theoretical basis on the structure and dynamics of insulin-cyclodextrin complex for new oral insulin formulation. Docking and molecular dynamics simulations were performed to explore the interactions between insulin monomer and insulin dimer with β-cyclodextrins (β-CDs). A multiple molecular docking study was performed using the Autodock v4.2 program to determine the number of β-CD that can adhere to the binding sites of insulin as well as to determine the most stable conformations of insulin to β-CDs. A 100 random structure docking using 1:1 insulin monomer-β-CD and insulin dimer-β-CD ratio were conducted and from the final docked structure, additional β-CDs were added and the process were repeated until the energy increase. Molecular docking results revealed that a maximum of four β-CDs can bind to an insulin structure with the 1:3 insulin-β-CD ratios having the lowest binding free energy. A 100 ns molecular dynamics simulation was then conducted to verify the results obtained by molecular docking. In addition to the hydrogen bonding formations, the docked conformations showed that hydrophobic interactions played a crucial role in insulin-β-CD conformational stability. The analysis of the molecular dynamics simulation confirmed the stability of the 1:3 insulin dimer-β-CD complex system with root mean square deviation (RMSD) values of 0.40 ± 0.02 nm after 38 ns and showed a large difference compared to the free insulin system. The increase

of solvent accessible surface area (SASA) values in 1:3 insulin dimer-β-CD complex is consistent with the hydrophobic interactions in the formation complex, therefore increasing the insulin folding and stability. The root mean square fluctuation (RMSF) profiles of the 1:3 insulin dimer in complex system also showed a reduced flexibility of amino acid residues of insulin dimer in complex compared to free insulin. Furthermore, the structure and dynamics of amino acids in insulin at each binding site along with its effect on β-CDs were further investigated. The results indicated that the β-CDs were stably bound to each binding site of the insulin dimer with limited movement around the mean value. In addition to this, the polar amino acids of cystein (Cys62), serine (Ser63) and glutamine (Gln66) were involved with the highest occupancy of hydrogen bonds with 74.0 %, 54.3 % and 51.9 %, respectively. The theoretical results indicated the presence of significant interactions between insulin and β-CD, which could provide useful insights into an oral insulin formulation.

CHAPTER 1

INTRODUCTION

1.1 Introduction

During the last decade, approximately 300 million people worldwide were affected by Diabetes Mellitus (DM) (Williams et al., 2002) with the Asian countries representing more than 60% of that diabetic population (Ramachandran et al., 2012). DM is defined as an endocrine disease which is related to the disorders of carbohydrate metabolism carried about by deficiency in insulin secretion, insulin resistance or both (Belchetz & Hammond, 2004). There are three types of DM: Type 1, Type 2 and the gestational diabetes that happens only to pregnant women (Carino & Mathiowitz, 1999).

Type 1 diabetes occurs due to the destruction of pancreatic beta islet cells. This leads to insulin deficiency and predisposes individuals to diabetic ketoacidosis (Mohan, 2005). This type of diabetes usually happens in childhood and requires patients to inject themselves with insulin to reduce glucose levels. Type 2 diabetes occurs in adults and is caused by the inability of the pancreas to produce insulin and resist peripheral insulin (Gale, 2001; Skyler, 2004). The development of factors leading to Type 2 diabetes are due to genetic inheritance, beta cell dysfunction (Mohan, 2005), the environment of beta cells (Pittas & Greenberg, 2003), insulin resistance (Saltiel, 2001), obesity (Kahn & Flier, 2000), the role of fatty acids which inhibit insulin signaling (Shulman, 2000), the role of adipokines (Saltiel, 2001), improper nutrition (Steyn et al., 2004) and low physical activity in patients (Caro et al., 1989).

In order to prevent these complications, people with Type 2 diabetes are usually treated with dietary measures, oral medicines and exercises. Insulin injections are compulsory for Type 1 diabetic patients, however, some Type 2 diabetic patients need insulin injections to control the glucose level in their blood. Due to the fast development in biotechnology over the past few decades, variants of insulin from different sources were produced. These include rapid (Shafie et al., 2014), short (Siebenhofer et al., 2006), intermediate (Norrman et al., 2007), long (Luzio et al., 2013) and ultralong-acting (Wang et al., 2012) insulin. These insulin variants must be administered via injections and may result in one or more disadvantages to patients such as needle phobia, skin bulges, allergic reactions, common infections and tenseness of long-term usage of injection insulin (Kennedy, 1991; Khafagy et al., 2007). Furthermore, the treatment of injected insulin can lead to peripheral hyperinsulinemia, which is due to the directly insulin transfer into general circulation in human body. Hyperinsulinemia is diabetes's side effect which is linked to cancer, hypoglycemia, peripheral hypertension and the development of atherosclerosis (Nordestgard, 1997). The clinical trials by Pamnani (2008) shows that a significant percentage of patients failed to achieve lasting glycemic control due to the patients' noncompliance with injectable insulin treatment. Due to this problem, current research on insulin has focused on the development of an alternative delivery method to injection. These include buccal/sublingual (Portero et al., 2007), nasal (Yu et al., 2004), pulmonary (Stephen et al., 2000), ocular (Owens, 2002), transdermal (Rastogi et al., 2010) as well as oral (Zhang et al., 2013).

Oral insulin administration is the most effective and possible technique to be used compared to the other routes. This is because insulin will be directly transferred to the liver and consequently avoids the peripheral hyperinsulinemic side

effects to patients caused by injection. Furthermore, the pain caused by injections and the psychological barriers to daily injections such as needle phobia can be eliminated via this oral route (Korytkowski, 2002).

However, there are major hurdles to the effective use of orally delivered insulin. These obstacles include the rapid enzymatic degradation of insulin in the stomach, inactivation and digestion by proteolytic enzymes in the intestinal lumen, low penetration rate of insulin through the gastrointestinal membrane as well as biological and structural stability of the insulin in a body system (Timmy et al., 2002; Elsayed, 2012). In order to overcome these barriers for successful oral administration, many research groups are currently working towards developing new oral insulin formulations. These include the attempt to overcome barriers and limitations through chemical modification of insulin with various fatty acids (Ashada et al., 1995), enzyme inhibitors and penetration enhancers (Liu et al., 2003; Miller & Johnston, 2005) and incorporation of insulin into carriers such as hydrogels (Nakamura et al., 2014), liposomes (Choudhari & Labhasetwar, 1994), erythrocytes (Al-achi & Greenwood, 1998), nanospheres (Damge et al., 1997) and nanocubicles (Chung et al., 2004).

A promising approach used in oral insulin delivery is the encapsulation of insulin by cyclodextrin (Zhang et al., 2010; Sajeesh et al., 2010; Uehata et al., 2011; Uehata et al., 2012; Zhang et al., 2012; Zhang et al., 2013). The complexations of insulin with cyclodextrins offer a unique and effective way to improve the insulin properties and stability against aggregation, thermal denaturation and degradation (Sigurjonsdottir et al., 1999; Dong et al., 2002). Previously, studies on the complexation of insulin with cyclodextrins focused on the interactions of the cationic

β-CD polymers with insulin (Huang et al., 2010), the insulin cell-penetrating peptide co-administrations with hydroxylpropyl-β-CD (Zhang et al., 2010), insulin glargine with maltosyl-β-CD (Uehata et al., 2012), insulin encapsulated polymethacrylic acid (PMAA) hydrogel microparticles with methyl-β-CD (Sajeesh & Sharma, 2006), insulin with hydroxylpropyl-β-CD (Zhang et al., 2009), kinetic degradation of insulin complexed with methyl-β-CD (Dotsikas & Loukas, 2002) and β-CD grafting hyperbranched polyglycerols as carriers for insulin (Zhang et al., 2011).

Computer modeling is widely used to understand and predict the properties and behaviour of systems at the molecular level. Molecular modeling of insulin using molecular dynamics method has been reported. These studies include the dissociation of insulin-phenol complex (Kru, 2003), binding of glucose to insulin (Falconi et al., 2001a; Zoete et al., 2004a), dynamic behaviour of insulin monomer and dimer (Zoete et al., 2004b), association of glycoprotein structure with B chain human insulin (Stavrakoudis, 2011), insulin stability on graphene (Liang et al., 2009), flexibility of B chain human insulin (Legge et al., 2006), structure and stability of insulin dimer (Falconi et al., 2001b), insulin interaction with boron-nitride and functionalized graphene nanosheets (Atabay et al., 2014), conformational flexibility of insulin analogs (Ksenofontova & Stefanov, 2013), simulation of insulin protection by trehalose (Li et al., 2014) and the aggregation and release rate of insulin (Berhanu & Masunov, 2012).

1.2 Problem Statement

Among the latest development in oral insulin delivery formulation, the complexation of insulin with cyclodextrin has been found to be advantageous and encouraging. The experimental data on the complexation of insulin with β-CD on varying conditions has been investigated in the past few years and support the fact that complexation with cyclodextrin improve the stability of insulin. However, it is not really understood on how cyclodextrins interact with insulin which may or may not lead to insulin conformational change and how these interactions affect the thermodynamics and structural properties of insulin upon binding to CD. In order to improve our understanding on the molecular properties insulin-β-CD formation, theoretical study comprising of quantum mechanics, molecular docking and molecular dynamics simulation was conducted as part of our attempt to understand the β-CD behaviour toward insulin monomer and dimer as an oral delivery medium for insulin.

1.3 Objectives

The objectives of this research are:

1. To determine the binding sites of β-CDs onto insulin monomer and insulin dimer and the best ratios of β-CDs to insulin using molecular docking calculation.
2. To determine the structural change and stability of insulin monomer and insulin dimer following the complexation with β-CDs using molecular dynamics simulation.

3. To identify the type of interactions exists between β-CDs and the amino acids of insulin involved in the stabilization of the insulin-β-CD complexes.

1.4 Thesis Outline

This thesis contains five chapters. Chapter 1 provides an overview and the background of this study. Chapter 2 reviews the structure, properties of insulin as well as cyclodextrins and the previous study on protein-cyclodextrin systems. The fundamentals behind computer modeling techniques applied in this study were also discussed in this chapter. Chapter 3 describes the methodology for the geometry optimization of single insulin monomer and insulin dimer as well as the β-CD structure, the multiple molecular docking and finally, the molecular dynamics simulation of insulin-β-CD conformations in detail. Chapter 4 contains the results and discussion which are divided into three main sections: geometry optimization, molecular docking results and molecular dynamics simulation study. Finally, Chapter 5 summarizes the research findings and further recommendation for possible research related to this study. Appendices associated with the relevant documents were provided at the end of this thesis.

CHAPTER 2

LITERATURE REVIEW

2.1 Diabetes and Insulin

Worldwide, it is expected that over 500 million people will be affected by DM in 2030 (Williams et al., 2002). DM causes about 5% of deaths each year (Liu et al., 2010) and this is likely to increase by more than 50% in the next 10 years without proper medication (http://www.who.int/diabetes/en/). The increasing prevalence of diabetes may be due to several factors which are population growth, ageing where an increased life expentancy results in a higher ratio of aged population more prone to diabetes, urbanisation, increasing obesity, increasing physical inactivity and passive lifestyles (Sonia & Sharma, 2014).

The word '*Diabetes*' comes from the Greek work for 'pipe-like' because essential nutrients of the body start to pass through the system instead of being utilised. Meanwhile *'Mellitus'* is the Latin word for 'honey' or 'sweet' (van Diepen, 1996). Scientifically, DM is a chronic metabolic disease, which is caused by insulin deficiency and an abnormal increase of blood sugar levels in the body (Genuth et al., 2003). Type 1 DM is most commonly diagnosed in children and adolescents, which cannot be prevented and thus occurs when the pancreas does not produce insulin at all or only a little. Meanwhile, Type 2 DM occurs when the pancreas produces too little glucose. In Type 2 cases, the production of insulin is normal initially, but the response activated by insulin in the peripheral tissues is blunted (Alexander & Hunter, 2004). When the production of insulin by the pancreas can no longer compensate for the peripheral insulin resistance due to β-cell dysfunction, the Type 2 DM and hyperglycemia become overt. This type of DM usually occurs with

increasing of age and depends on the lifestyle of the individuals. Type 2 diabetes is also found as a component of metabolic syndrome, which is characterised by hypertension, central obesity, hyperlipidemia, and insulin resistance that results in increased mortality due to cardiovascular incidents. The pervasiveness of both Type 2 diabetes and metabolic syndrome is reaching epidemic proportions, as the onset average age of both diseases has markedly decreased over the past decades (Horton, 2008).

Currently, there is no practical cure for diabetes. However, it can be controlled effectively through several ways. Lifestyle change involving a modified dietary sugar intake and physical exercise (Franz, 1997) should be taken into consideration. The oral hypoglycaemic agents are administered to treat diabetic patients when diet and exercise are not enough to achieve the desired glycaemic control (Krents & Bailey, 2005). There are four classes of hypoglycaemic agents; insulin secretagogues (Dunning, 1997), insulin sensitizers (Bailey et al., 1992), α-glucosidase inhibitors (Gerard et al., 1984) and insulin. Insulin secretagogues, insulin sensitizers and α-glucosidase inhibitors are oral medication treatments for stimulating endogenous insulin by the pancreas. Meanwhile, insulin is a protein therapy needed to be administered exogenously (Sonia & Sharma, 2014).

As a protein therapy used to treat diabetes, insulin was widely used to regulate the level of glucose in the blood system. Approximately 20–30% of all diabetic patients receive daily insulin injections in order to maintain their glucose levels (Babu et al., 2008). Human insulin is synthesised from beta-cells of the islets of Langerhans and is secreted into the bloodstream (Greenspan & Gardner, 2004). It plays a crucial role in monitoring the metabolic activities of the body, particularly the

homeostasis of the blood glucose. Insulin secretion is a regulated process providing a stable concentration of glucose in blood during eating and fasting (Yeh et al., 2010).

The history of insulin as a protein therapy to diabetes started back to 1922 when it was first used successfully in humans to treat the symptoms of DM (Bliss, 1993). The study of diabetes, including the related aspects of glucose metabolism was also a fertile ground for scientific inquiry that 10 scientists received the Nobel Prize for diabetes-related investigations between 1923 to 2014 as shown in Table 2.1 (Kim, 2011). In 1958, Frederick Sanger was awarded the Nobel Prize for developing techniques to sequence the amino acids of insulin.

Rosalyn Yalow in 1960 permitted the quantitative measurement of pancreatic beta-cell function in animals and humans and established the radioimmunoassay as a powerful tool for measuring proteins, metabolites, and other chemicals present in very low concentrations. After that, Donald Steiner's in 1967 demonstrated two polypeptide insulin molecules were derived from a single chain precursor proinsulin. This formation was crucial for the understanding of the biochemistry of insulin and was also applied to other peptide hormones. The crystal structure of insulin was then determined using 3D crystallography technique by Dorothy Hodgkin in 1969. Insulin was the first hormone to be cloned and then produced for therapeutic use by means of recombinant DNA technology, while providing an unlimited supply of this important molecule and laid down the foundation for the biotechnology industry (Ullrich et al., 1977). In the recent development in insulin, Karplus won the Nobel Prize for developing multiscale models to describe complex biomolecules. Karplus and co-workers successfully developed methods which combined quantum and classical mechanics to describe the interaction between glucose and insulin (Zoete et al., 2004a).

Table 2.1

Nobel Prizes for Diabetes Related Research (Kim, 2011)

Year	Recipient	Contribution
1923	Banting and Macleod	Discovery of insulin
1947	Cori and Cori	Discovery of the course of the catalytic conversion of glycogen
1947	Houssay	Discovery of the role of hormones released by the anterior pituitary lobe in the metabolism of sugar
1958	Sanger	Discovery of the protein structures including insulin
1960	Yalow	Development of radioimmunoassays for peptide hormones
1969	Hodgkin	Discovery of insulin crystal structure using 3D crystallography technique
1971	Sutherland	Discovery of the mechanisms of action of hormones
1992	Fischer and Krebs	Discovery of reversible protein phosphorylation as a biologic regulatory mechanism
2014	Karplus	Development of multiscale models for complex chemical systems including insulin

2.1.1 Structure of Insulin

Insulin is synthesised in beta cells of the pancreas where the messenger RNA (mRNA) is translated as a single chain precursor named preproinsulin (Dodson & Steiner, 1998). The removal of preproinsulin's signal peptide during insertion into the endoplasmic reticulum (ER) generates proinsulin. After that, the proinsulin folds into a three-dimensional structure, whereby it is transported with the help of vesicles to Golgi. In this medium, proinsulin is likely self-assembles to form proinsulin hexamers, in which connecting peptide linking A and B chain, then is cleaved by enzymes. This promotes towards the formation of zinc assembled of insulin hexamer, which are capable of forming microcrystals. Finally, hexamers will fall apart into insulin monomers due to repulsion in certain charged residues at its core (Vashisth, 2010). The final formation of insulin is in monomeric form which is used to stabilise glucose concentrations in living system.

Insulin monomer is a small globular protein composed of two polypeptide chains. A Chain consists of the sequence of 21 amino acids, whereas B chain consists of 30 amino acids. Both chains were stabilised by three disuplhide bonds, whereby, two disulphide bridges between A chain and B chain were formed between the residues Cystein7 (Cys7) to Cystein28 (Cys28) and Cystein20 (Cys20) to Cystein40 (Cys40). The other disuphilde bridge was internally formed between residues Cystein6 (Cys6) to Cystein11 (Cys11) in A chain of insulin monomer. Generally, A chain contains N-terminal α-helix, turn conformations, whereas the B-chain contains N-terminal and central α-helix, and C-terminal β-strand (Olsen et al., 1996). At micromolar concentration, the insulin dimerises and further forms hexamers in the presence of zinc and phenolic species as shown in Figure 2.1.

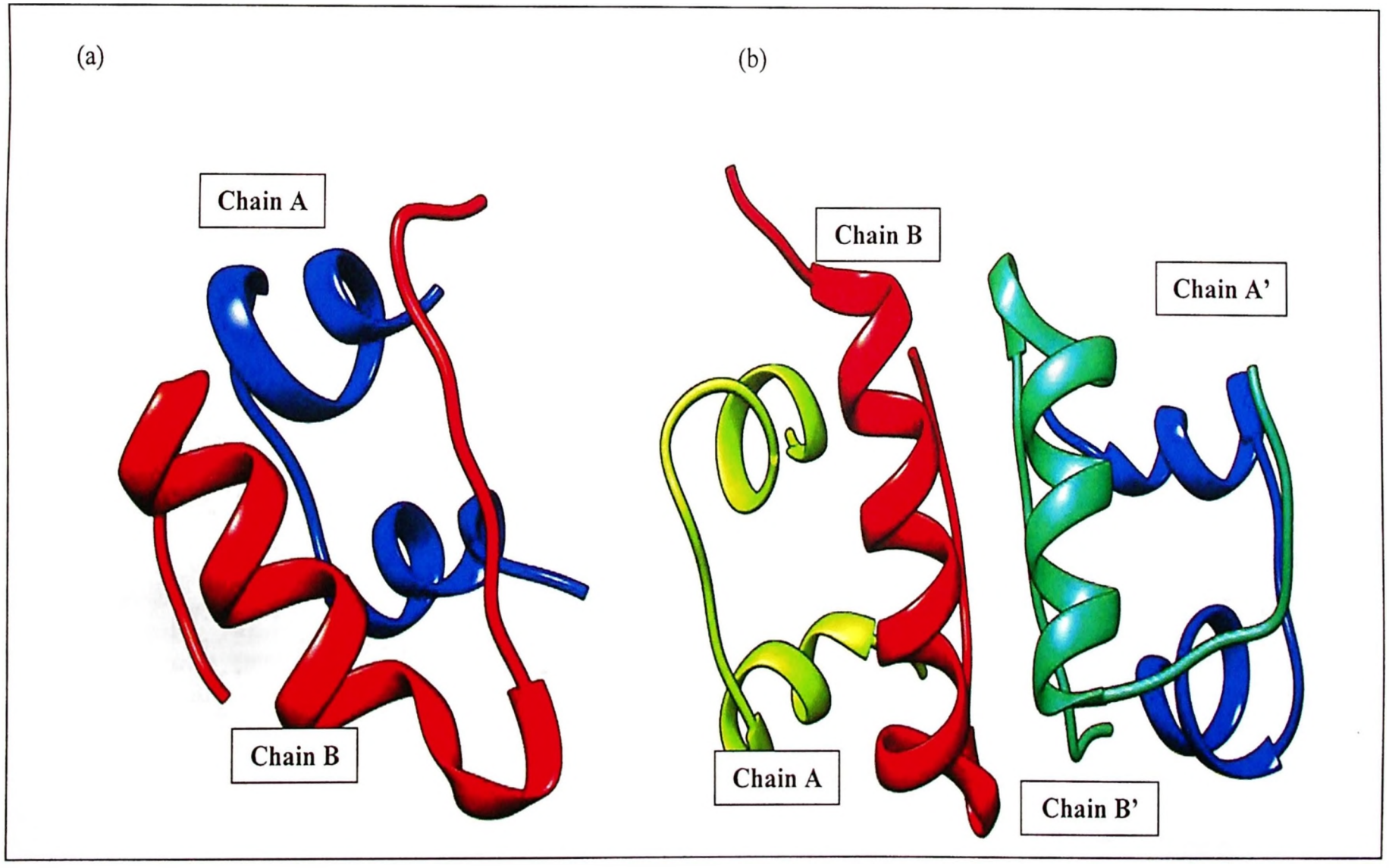

Figure 2.1. Structures of insulin (a) monomer (b) dimer.

The insulin dimer was formed when the extended C-terminal of two monomers are brought together, thus, forming a two-stranded antiparallel β-sheet. The interactions stabilizing the dimer form of insulin are predominantly non-polar with β-sheet hydrogen bonds replacing water hydrogen bonds and contributing to orientate two monomers (Jorgensen et al., 1996). The published high resolution X-ray structures of insulin suggested as the aggregated species at which several reports provide the results for monomeric form of insulin (Zhang et al., 2002). Therefore, the current insulin structure was derived from insulin hexamer and dimer crystal structures.

Biologically, insulin monomer is active and capable of self-association forming dimers and hexamers in biosynthesis and during storage of insulin (Dodson & Steiner, 1998). Insulin monomer is unstable compared to dimer and hexamers and is therefore the main reason why many researchers used the dimer form. In addition, insulin monomer forms aggregates and fibrils due to the interactions between the hydrophobic residues in the monomer and this behaviour are often associated with the reduction of biological potency of insulin monomer (Dunn, 2005). Insulin dimer and hexamer are also known to be more resistant to chemical and physical degradation than its monomer form (Loftsson et al., 2005). Insulin monomer with A and B chains comprises of Gly1 → Ala51, while, insulin dimer is made up of two monomers with chains A, B, A' and B' consists of Gly1 → Ala102 as shown in the numbering structure of insulin in Figure 2.2.

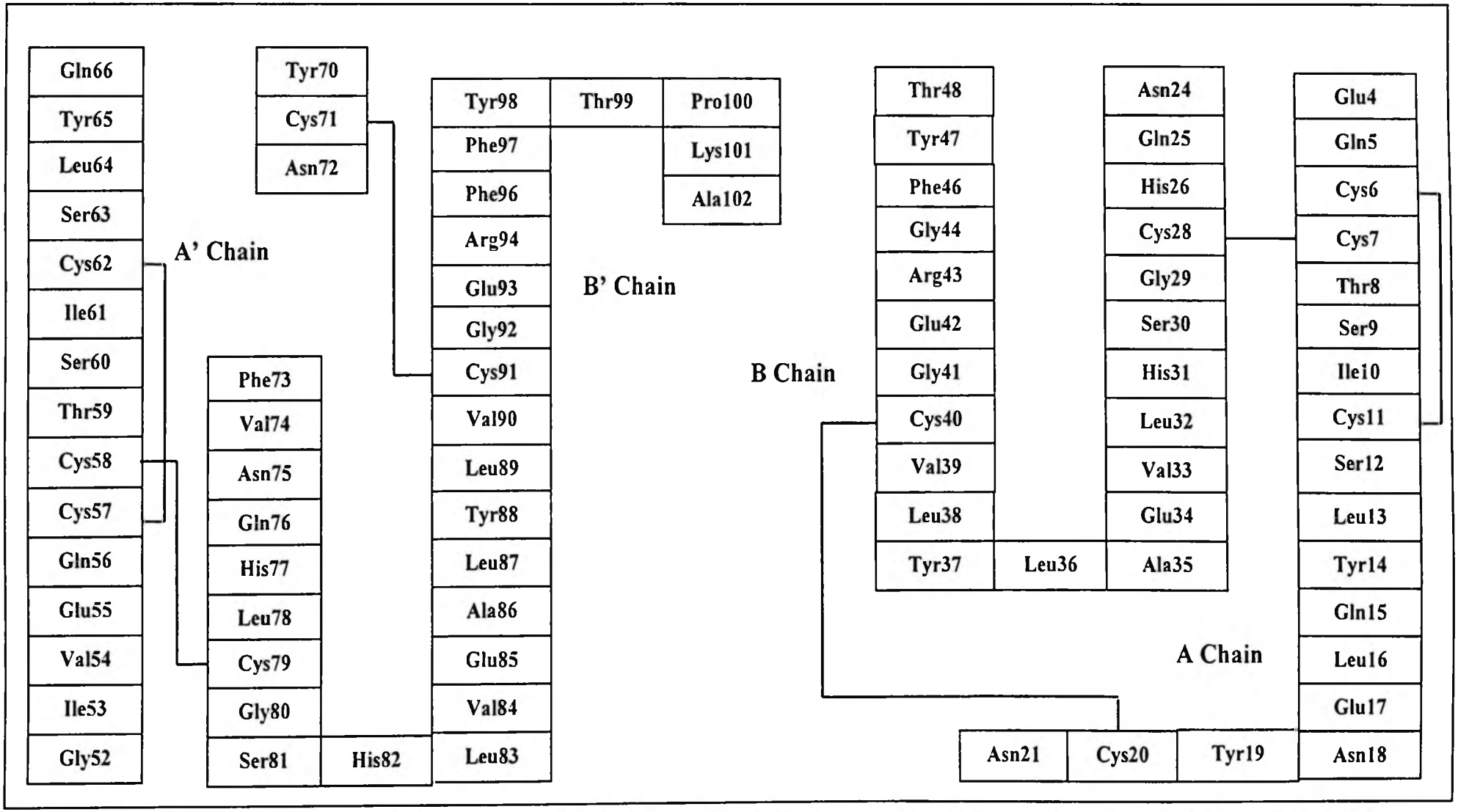

Figure 2.2. The numbering scheme for insulin dimer, which comprises of monomers I and II. Blue lines represent the disuplhide bridges.

2.1.2 Development of Insulin Delivery

The most crucial treatment strategy for DM focuses on the control of postprandial blood glucose. Therefore, the goal of exogenous insulin is to mimic the physiological profile in a non-diabetic person. In the past few decades, various routes of insulin administration were explored in order to discover a new technique of insulin administrations that will improve the metabolic effects of insulin. The current administration of insulin which is via subcutaneous route has various disadvantages, such as injection site pain, low patient compliance, and occasional hypoglycemia (Khafagy et al., 2007). Furthermore, insulin administration via subcutaneous injection does not mirror the normal dynamics of endogenous insulin release, promoting in failure to achieve lasting glycemic control (Hoffman & Ziv, 1997; Morishita et al., 2006). Various routes other than subcutaneous, which under investigation for insulin delivery include oral (Huang et al., 2009; Zhang et al., 2013), pulmonary (Mastrandrea, 2010), transdermal (Krishnankutty et al., 2009), nasal (Turker et al., 2004), buccal (Senel & Hincal, 2001), ocular (Lee et al., 2002), rectal (Ritschel et al., 1988) and vaginal (Choudhury et al., 2011). Among all the possible routes, oral insulin delivery serves as the most expedient and desired way of insulin administration due to patient compliance and comfort. Furthermore, an oral pathway of insulin delivery is expected to follow the physiological route of insulin secretion (Lewis et al., 1996), which is accompanied by greater hepatic versus peripheral concentrations (Gordon-Still, 2002).

There are a few other major hurdles to develop oral insulin delivery, such as the presence of chemical and enzymatic barrier in the gastrointestinal tract that could degrade the insulin structure (Sonia & Sharma, 2014), the poor permeation of insulin

across the intestinal epithelia (Muller, 2011), with low bioavailability (typically less than 1–2%) (Renukuntla et al., 2013), and stability of insulin which depend on a few factors such as temperature, pH, solvent, solutes and crystallinity states of the insulin (Manning et al., 1989). One of other major barrier in order to develop oral delivery insulin is poor of insulin absorption through the gastrointestinal membrane (Carino & Mathiowitz, 1999). The high molecular weight of insulin, which is about 6 kDa could not also penetrate through this route. The insulin absorption is prevented due to its large molecule size, charge and hydrophilicity (Elsayed, 2012). Oral delivery of insulin for DM was tried in diabetic induced rats by Roques and co-workers (1992) who reported one of the major problems is degradation of the insulin due to proteolytic activity of enzymes in the gastrointestinal tract.

A perfect oral drug delivery system should be capable of maintaining the purity of insulin molecules until it reaches the absorption site, releasing insulin at the targeted absorption site and maintaining inside the gastrointestinal tract irrespective of its transitory constraints (Renukuntla et al., 2013). The crucial parts of oral delivery insulin should be safe delivery to human body, increased in bioavailability as well as enhanced insulin absorption. Many research groups focused on development of a delivery system for insulin oral administration using absorption enhancers (Onuki et al., 2000; Thanou et al., 2000), enzyme inhibitor (Agarwal et al., 2000), enteric coatings (Hosny et al., 2002) and nanoparticle delivery (Ezpeleta et al., 1999). The permeation of nanoparticles still results in poor bioavailability of the insulin. The use of protease inhibitors in addition to absorption enhancers leads to improved uptake insulin. Nonetheless, these agents are not specific and could assist in the uptake of other unwanted protein or peptides in the gastrointestinal tract

(Nakamura et al., 2014). Some promising results were also obtained, indicating clearly that oral administration of insulin mimicking the physiological fate of insulin. However, most production techniques involve the use of organic solvents, heat or vigorous agitation potentially harmful to the structure as well as the biological activity of insulin and were lead to problems of cytotoxicity (Khafagy et al., 2007). These limitations could be effectively overcomed by insulin encapsulation agent such as β-CD and derivatives (Ahsan et al., 2003; Sajeesh et al., 2010; Zhang et al., 2012).

2.2 Cyclodextrins

2.2.1 Structures and Properties of α-, β- and γ-CDs

Cyclodextrins (CDs) are cyclic oligosaccharides linked through α-1, 4-glycosidic bonds which composed of 6, 7 or 8 glucose units classified as α-, β-, and γ-CDs, respectively, as shown in Figure 2.3. CDs are produced from enzymatic digestion of starch by CD glycosyltransferase (CGTase) isolated from *Bacillus maceranc* (French, 1957). Due to advancement of biotechnology in recent decades, highly purified CD and CD derivatives were widely produced in large scale (Loftsson & Duchene, 2007).

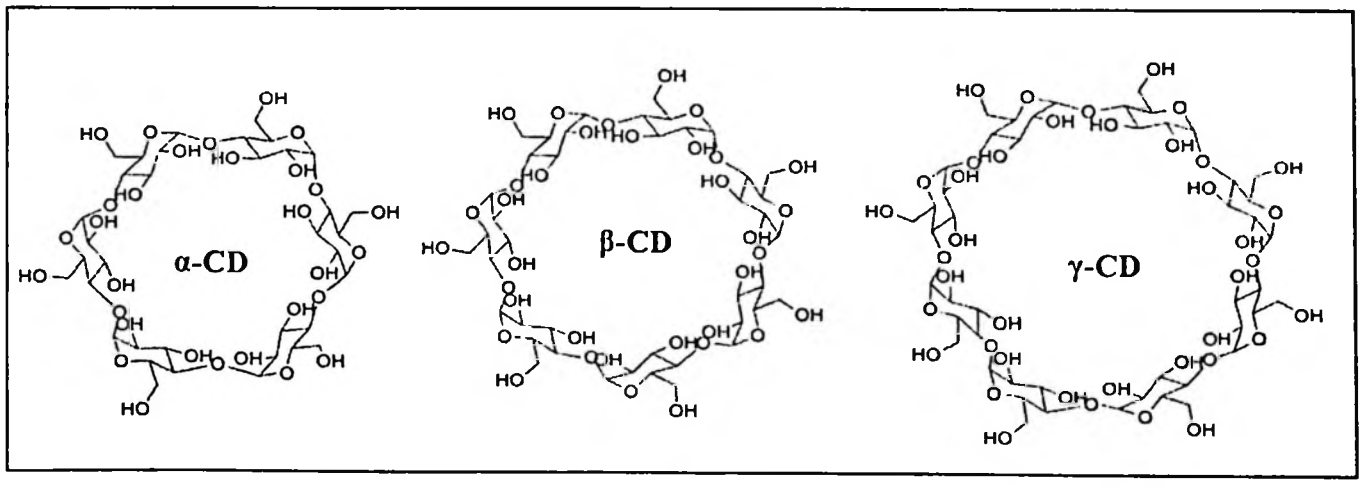

Figure 2.3. Structures of α-, β- and γ-CDs (Szejtli, 1998).

Each of the chiral glucose units in CD is in the $^4C_1$ chair conformation of the sugar units, providing a hollow truncated cone shape where hydroxyl groups are oriented to the exterior, while skeletal carbon and ethereal oxygen moieties are oriented to the central cavity (Brewster & Loftsson, 2007). The C-2-OH group of one glucopyranose unit can form a hydrogen bond with the C-3-OH group of the adjacent glucopyranose unit. A complete secondary belt is formed by this hydrogen bonding formation, thus providing a rigid CD structure. The interior cavity of CD is hydrophobic, while the exterior site is hydrophilic in nature (Connors, 1997) as shown in a schematic view in Figure 2.4.

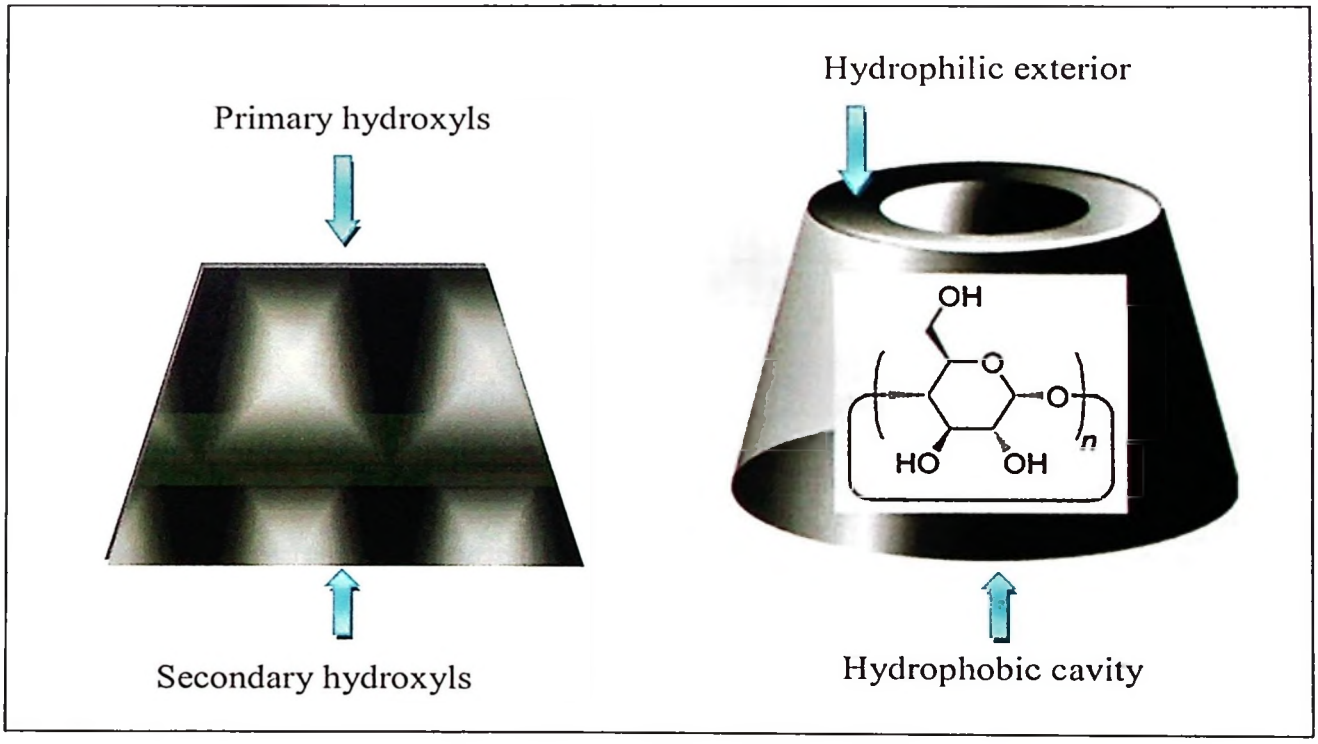

Figure 2.4. The hollow truncated cone structure of CD.

The cavity size of α-CD is the smallest of the three CDs while γ-CD has the largest cavity size of all three CDs and is the most expensive too. Therefore, β-CD is

Each of the chiral glucose units in CD is in the $^4C_1$ chair conformation of the sugar units, providing a hollow truncated cone shape where hydroxyl groups are oriented to the exterior, while skeletal carbon and ethereal oxygen moieties are oriented to the central cavity (Brewster & Loftsson, 2007). The C-2-OH group of one glucopyranose unit can form a hydrogen bond with the C-3-OH group of the adjacent glucopyranose unit. A complete secondary belt is formed by this hydrogen bonding formation, thus providing a rigid CD structure. The interior cavity of CD is hydrophobic, while the exterior site is hydrophilic in nature (Connors, 1997) as shown in a schematic view in Figure 2.4.

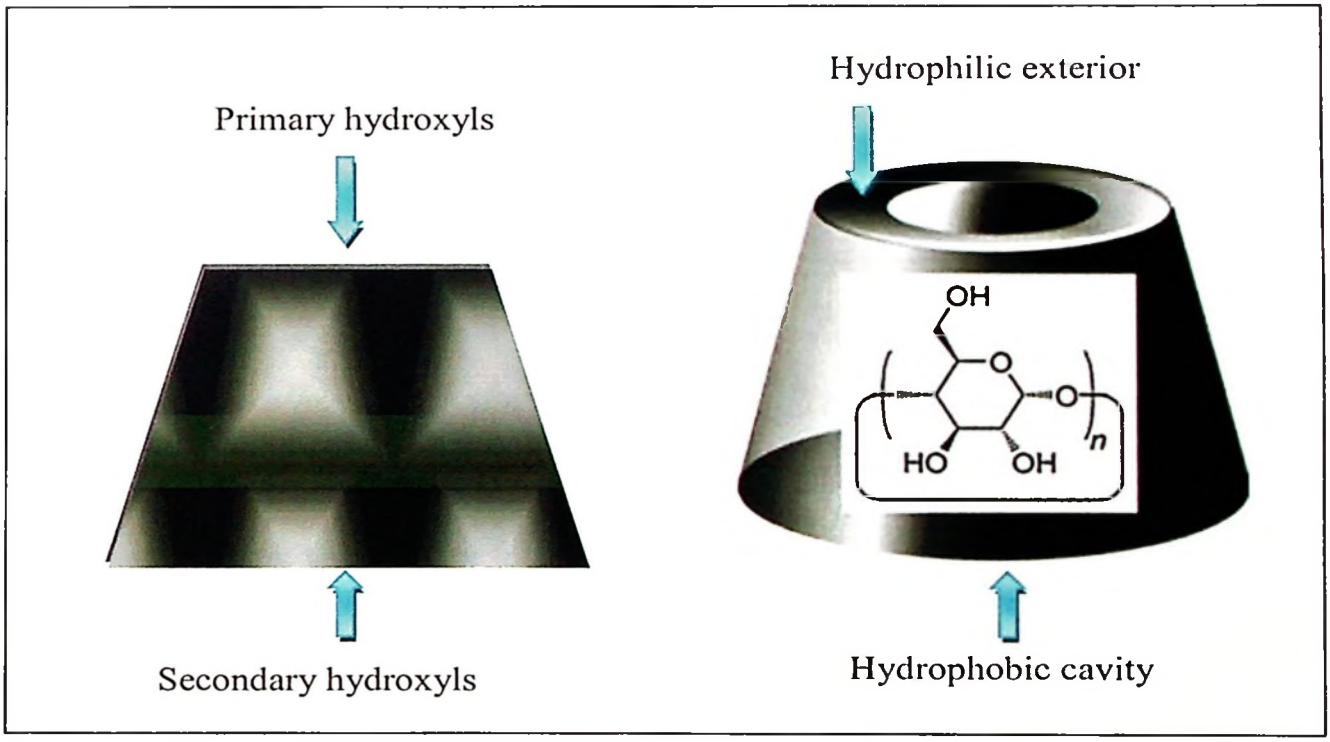

Figure 2.4. The hollow truncated cone structure of CD.

The cavity size of α-CD is the smallest of the three CDs while γ-CD has the largest cavity size of all three CDs and is the most expensive too. Therefore, β-CD is

most widely used in research and manufacturing due to its cost and suitable cavity size for most compound molecules (Loftsson & Brewster, 1996; Szejtli, 1998).

Cyclodextrins are soluble in water and insoluble in most organics solvents although β-CD is the least soluble in water compare to the other native CDs. The solubility of cyclodextrins in water is unusual and does not seem to be dependent on the structure of cyclodextrin (Sabadini et al., 2006). α-CD is the most strained torus structure while γ-CD ring is the least strained among the three native cyclodextrins. β-CD is 9 and 11 times less soluble in water in comparison to α-CD and γ-CD, respectively. Table 2.2 summarizes the size and the properties of native CDs (Szejtli, 1998).

Table 2.2

Physicochemical Properties of α-, β- and γ-CDs

Properties/CD	α-CD	β-CD	γ-CD
Number of glucose units	6	7	8
Molecular weight	972	1135	1297
Solubility in H_2O (g/mL)	14.50	1.85	23.20
pK_a	12.33	12.20	12.08
Inner diameter (Å)	4.70-5.30	6.00-6.50	7.50-8.30
Outer diameter (Å)	14.60 ± 0.4	15.40 ± 0.4	17.50 ± 0.4
Height (Å)	7.90 ± 0.1	7.90 ± 0.1	7.90 ± 0.1
Cavity volume ($Å^3$)	174	262	427

According to the report summarized by Szejtli (1998), CDs are mostly nontoxic. One of the few toxicities of CD reported is hemolysis, which is a phenomenon resulted from interactions of CD with membrane components (Szejtli, 1988; Shen et al., 1998). It is reported that lower concentration of CD protects the human erythrocytes against osmotic and heat-induced hemolysis. CD is known to cause the release of cholesterol and phospholipids from cell membrane thus resulting in cell disruption (Uekama et al., 1981; Shen et al., 1998).

Apart from that, the nonbonding electron pairs of the glycosidic oxygen bridges of β-CDs are directed toward the inside of the cavity producing high electron density and also lending on some Lewis base characteristics which is important for their capability to form host-guest complexes (Szejtli, 1998).

2.2.2 Applications of CDs

The modification of compounds with CDs lead to a large number of applications in various fields related to pharmaceutical industry, food technology, analytical chemistry, chemical synthesis and catalyst (Saenger, 1980; Szejtli, 1988; Wilsom & Verall, 1998) as illustrated in Figure 2.5.

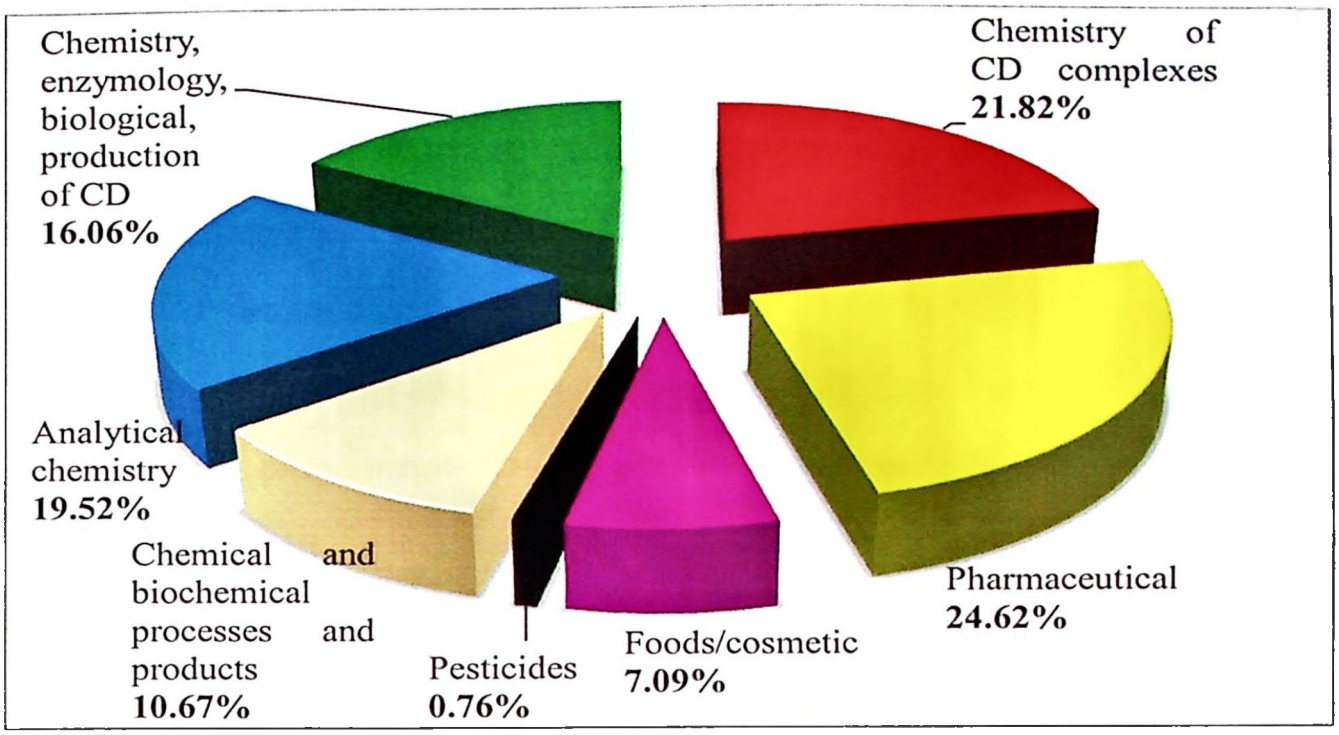

Figure 2.5. Distribution of the CD relevant abstracts published by *Cyclodextrin News* (*Cyclodextrin News*, 1996).

CDs provide many beneficial improvements to products including solubility and stabilisation of the guest compound which provide long-term protection of colour, odour and flavour of products. Besides, the nontoxicity of CD is also the main reason for its application in the pharmaceutical and food industry (Omari et al., 2010; Misiuk & Zalewska, 2011) with more than 40 formulations containing CDs on the market currently, intended for oral, parenteral, ophthalmic, rectal and dermal route of application (Gidwani & Vyas, 2015). Barone et al. (1998) and Nasongkla et al. (2003) reported that β-CD was used to increase bioavailability of poorly soluble drugs. Light, thermal and oxidative stability of drug molecules can also be improved through the formation of cyclodextrin complexes (Cwiertnia et al., 1999; Tirucherai & Mitra, 2003). Other pharmaceutical application of CDs is to reduce dermal (Uekama et al., 1992), ocular irritation (Loftsson & Stefansson, 1997) and to avoid adverse drug-ingredient interactions (Redenti et al., 2001).

One of the uses of CD in the cosmetic industry is in the production of long-lasting fragrances (Schmid, 1989) and reducing body odours (Trinh et al., 1999). Other applications include its use in toothpaste, skin creams, liquid and solid fabric softeners, paper towels and tissues (Szejtli, 1998) that contributed to 7.09 % of total use of CDs. In the food industry, β-CD was used to remove cholesterol from products such as milk, butter and eggs (Singh et al., 2010; Kemelbekov et al., 2011). Furthermore, the applications of CDs in agricultural fields include in the production of insecticides, fungicides and herbicides to improve plant growth and increase harvest yield. Other than that, CDs can also be used in many chromatographic techniques for separation of enantiomers (Szejtli, 1998). Table 2.3 summarises the applications of CDs in various sectors.

Table 2.3

The Summary Applications of CDs (Valle, 2003)

Sector	Applications
Cosmetic, personal care and toiletry	• Production of toothpaste, skin creams, paper towel, liquid and solid fabric softener, tissues, underarms shields, dishwashing and laundry detergent, talcum powder, sunscreen lotions.
Foods and flavours	• Removing cholestrols from milk, butter and eggs. • Improving texture on meat and pastry products. • Protecting the flavour throughout many rigorous food-processing methods of freezing, thawing and

	microwaving. • Production of dairy products with low cholestrols. • Improving elasticity in noodles, pie, dough and pizza. • Act as the antimicrobial food preservatives.
Pharmaceuticals	• Production of mouthwash solution, nasal drug, eye drop solution. • Reducing the effects of bitter or irritant tasting and bad smelling drugs.
Agriculture and chemical industries	• Production of herbicides, insecticides, fungicides, repellents, pheromones. • Use in separation of isomers and enantiomers. • Removing or detoxifying waste materials.
Adhesives, coating and polymers	• Increasing tackiness and adhesion of hot melts and adhesive.

2.2.3 Mechanism of Protein-CDs Interaction

Interactions between CD and protein have also given surprising outcomes as CDs were employed as catalyst to alter some properties of protein (Hamilton et al., 2000; Mcgarraghy & Darcy, 2000) and is in the improvement on protein solubility, which could lead to the protein stabilisation in water (Koralewska et al., 2004). This happened as the hydrophobic cavity of CDs is capable to provide temporary asylum for hydrophobic parts of protein molecules to dissolve in water. The solubilisation

ability of CDs is currently of interest in the development of many formulations to improve the bioavailability of protein system (Duchene, 1991).

Furthermore, the interactions of CDs with hydrophobic groups on protein molecules can reduce protein aggregation. This phenomenon was observed in the dissociation of bovine insulin dimers in the presence of different CDs (Lovatt et al., 1996). The inhibition of protein aggregation also occurred to bovine insulin (Dotsikas & Loukas, 2002), recombinant human growth hormone (rh-Gh) (Otzen et al., 2002) and several other proteins (Sharma & Sharma, 2001; Sigurjonsdottir et al., 1999). The study of Tavornvipas and co-workers (2004) showed the relationship between reduction of aggregation and binding constants of CD-derivatives and rh-GH. In their study, the branched CDs turned out to be the most competent in the prevention of protein unfolding and aggregation. The same CDs have been also reported to produce the highest stability constants among other CDs. Other findings involving the interaction between CD and protein include the binding mechanism between aripiprazole (APZ) with human serum albumin (HSA) in the presence of three types of CDs (Yan et al., 2015). The study reported the refolding recovery of APZ and HSA caused by CD which is specifically involved the interaction between HSA and CD. Current studies on the interaction between CD and protein such as phenylalanine dehydrogenase enzyme (Gubica et al., 2015) and mithochondrial ADP/ATP carrier (AAC) (Rather et al., 2015) have also been reported.

Nonetheless, proteins such as insulin are mostly hydrophilic and too bulky to be wholly encapsulated into the β-CD cavity. According to Irie and Uekama (1999) the hydrophobic side chains of insulin peptides penetrate into the β-CD cavity leading to the formation of non-covalent inclusion complexes. The CDs' ability to