Development of cyclodextrin stationary phases via 'click' chemistry for chiral capillary electrochromatography and high performance liquid chromatography

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**DEVELOPMENT OF CYCLODEXTRIN STATIONARY
PHASES VIA “CLICK” CHEMISTRY FOR CHIRAL
CAPILLARY ELECTROCHROMATOGRAPHY AND
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY**

**WANG YONG
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Summary

Due to increasing demand for enantiomerically pure (enantiopure) compounds, especially those of pharmaceutical importance, the development of stereoselective separation technologies has been attracting great attention during last decades. Direct enantioseparation by chiral stationary phases (CSPs) using chromatographic methods remains as the most important technique for chiral analysis as well as for preparative-scale chromatography. The development of novel stable and powerful CSPs is therefore important. High performance liquid chromatography (HPLC) is the most widely used separation technique and will continue to play an important role due to its maturity and easy operation. More recently, capillary electrochromatography (CEC), a new hybrid technology based on HPLC and capillary electrophoresis (CE), has been demonstrated to be a powerful alternative to chromatographic methods with several advantages over HPLC and CE.

There are several interrelated objectives in this dissertation project. Firstly, a new synthetic methodology was developed for the preparation of cyclodextrin (CD) based CSPs via copper (I) catalytic 1,3-dipolar cycloaddition reaction (“click” chemistry). The second objective has been to fabricate chiral capillary columns with selected CD CSPs for CEC enantioseparation and investigate the effects of CEC parameters on the separation. Finally, the enantio-recognition abilities of the obtained CD CSPs were fully investigated for enantioseparation of a large variety of racemic compounds in reversed phase HPLC.

The first part of this study involved a facile and reliable preparation of CD CSPs via “click” chemistry. Three monoazido-CD and two heptakisazido-CD derivatives were synthesized. Model reaction was implemented by “click” chemistry coupling of the monoazido-CDs with ethyl propiolate. An alkynyl modified silica was then prepared by refluxing synthesized N-3-(triethoxysilyl)propyl-2-propynamide in toluene. The desired CSPs were thereafter readily prepared in dimethylformamide (DMF) by “click” immobilization of the azido CDs onto the alkynyl silica catalyzed by soluble cuprous iodide-triphenylphosphine complex [CuI(PPh₃)]. Three CSPs (native CD **CSP1**, permethylated-CD **CSP2**, and perphenylcarbamoylated-CD **CSP3**) were prepared with a single triazolyl linkage and two CSPs (2,3-di-O-CD **CSP4** and 2,3-di-O-phenylcarbamoylated-CD **CSP5**) with multiple triazolyl linkages with relatively good surface coverage.

An effective methodology for CEC capillary fabrication and enantioseparation has been realized. Briefly, the capillary columns were packed with CSPs using slurry packing method followed by the successful fabrication of stable and penetrative sintered frits. The packed capillaries were conditioned for at least one hour with mobile phase driven by electroosmotical flow (EOF) before separation. On separation, the capillary was pressurized at both the ends and a voltage ramp time should be set to avoid bubble formation. In this study, three capillary columns were fabricated with **CSP1-3** for resolving some neutral racemic aryl alcohols and flavonoids. Consistent cationic EOF was obtained with acetonitrile (ACN) and phosphate buffer by solely using EOF as the driving force. The influences of

applied voltage, organic modifier content and buffer pH on the EOF and enantioseparation were evaluated. It was found that ACN/ phosphate buffer (5 mM pH=7) with a voltage of 5 kV was the optimum separation condition for **CSP3** packed capillary. Meanwhile, some drawbacks like low success ratio for capillary fabrication, difficulty in storage and short life of the packed capillaries were also encountered with this technique.

The enantiomeric resolution capabilities of **CSP1-3** were fully investigated for the separation of a wide range of racemic molecules in HPLC. Different CD functional groups render corresponding CSPs quite different recognition abilities. Native CD **CSP1** well separated dansyl amino acids and carboxylic acids, permethylated CD **CSP2** best resolved ionones while perphenylcarbamoylated CD **CSP3** possessed the most powerful resolving ability to racemic aryl alcohols. Furthermore, the chiral recognition ability of the CSPs could be largely tuned by altering the organic modifier types and proportion. Over fifty racemic compounds could be resolved with the three novel CSPs by optimizing the separation conditions. Good stability and separation results of the novel CSPs affirmed the reliability of triazolyl linkages in the CD CSP preparation. Compared with CEC, although lower chiral selectivity (α) and resolution (R_s) were found with HPLC, the steel columns were much easier to prepare, and instrument operation was quite simple. It should be mentioned that the two techniques each have their own advantages and disadvantages.

Efficient HPLC enantiomeric resolutions of over forty racemic compounds on

CSP4 and **CSP5** which have multiple triazolyl linkages were also performed. The free hydroxyl **CSP4** better resolved ketoprofen, Tröger's base, hydroxyl, carboxylic and dansyl amino acids while phenylcarbamoylated **CSP5** showed accentuated recognition ability to aryl alcohols, flavonoids, β -blockers and β -agonists. By comparison of the separation results on **CSP1** with single triazolyl linkage, it was found that different inclusion modes would take place for different analytes. The 2-OH, 3-OH and 6-OH groups on the CD rims acted distinctively in H-bond formation according to the analytes' structures.

Publication list

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Abbreviations

ACN	Acetonitrile
CD	Cyclodextrin
CE	Capillary Electrophoresis
CEC	Capillary Electrochromatography
CSP	Chiral Stationary Phase
DCC	<i>N,N'</i> -Dicyclohexylcarbodiimide
DMF	Dimethylformamide
Dns	Dansyl
ETCD	1-(6 ^A -Deoxy-β-CD)-4-(ethoxycarbonyl)-1,2,3-triazole
ETMeCD	1-(6 ^A -Deoxy-permethylated-β-CD)-4-(ethoxycarbonyl)-1,2,3-triazole
ETPhCD	1-(6 ^A -Deoxy-perphenylcarbamoylated-β-CD)-4-(ethoxycarbonyl)-1,2,3-triazole
FTIR	Fourier Transform Infrared Spectroscopy
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
heptakis-N ₃ -CD	Heptakis(6-azido-6-deoxy)-2,3-di-O-β-CD
heptakis-N ₃ -Ph-CD	Heptakis(6-azido-2,3-di-O-phenylcarbamoylated)-β-CD
MeOH	Methanol
Me-N ₃ -CD	Mono(6 ^A -azido-6 ^A -deoxy)-permethylated-β-CD
MS	Mass Spectrometry
N ₃ -β-CD	Mono(6 ^A -azido-6 ^A -deoxy)-β-CD
NMR	Nuclear Magnetic Resonance
NPs	Nanoparticles
Ph-N ₃ -CD	Mono(6 ^A -azido-6 ^A -deoxy)-perphenylcarbamoylated-β-CD
SFC	Supercritical Fluid Chromatography
TEA	Triethylamine
TEAA	Triethylammonium acetate
TsO-β-CD	Mono(6 ^A -tosyl)-β-CD
UPLC	Ultra Performance Liquid Chromatography

Chapter 1

Introduction

1.1 Chirality, enantiomeric separation and principles

1.1.1 Chirality

Chirality, or handedness, is of key importance in many branches of science. An object that cannot be superimposed with its mirror image is said to be chiral. Examples would include human hands, feet and many kinds of molecules (Figure 1-1). In chemistry, chirality usually refers to molecules. Two mirror images of a chiral molecule are called enantiomers or optical isomers. The most important nomenclature system for denoting enantiomers is the *R/S* system. Absolute configuration of the isomers are performed by labeling each chiral center *R* or *S* according to a system by which its substituents are each assigned a priority, according to the Cahn-Ingold-Prelog priority rules (CIP), based on atomic number [1]. Chirality can result from a variety of sources aside from the single chiral carbon or chiral center. It should be noted that it is unnecessary for a molecule to possess a chiral center in order to exist in enantiomeric forms, but of necessity to be non-superposable with its mirror image as a whole.

Figure 1-1 Chiral objects and molecules

Chiral molecules are found in all living things [2]. Proteins, nucleic acids, amino acids and polysaccharides possess chiral structures and are building blocks to life. Chirality plays an essential role especially in the pharmaceutical industry [3-6]. A great percentage of pharmaceutical compounds and those under research are enantiomers, many of which show significant differences in their pharmacokinetics and pharmacodynamics characteristics [7-9]. Using ethambutol and thalidomide as examples, one enantiomer of ethambutol is used to treat tuberculosis while the other isomer causes blindness; *R*-thalidomide is a sedative and effective against morning sickness, whereas its analog is teratogenic. Even if the side effects of many enantiomers are not drastic, an unnecessary burden for the organism will be caused due to the metabolism of the inactive enantiomer. A guideline was issued in 1992 by US Food and Drug Administration (FDA) that each drug enantiomer must be studied separately for its pharmacological pathways, and that only therapeutically active drug isomer should be marketed [10]. The sales of chiral fine chemicals as single isomers was \$6.63 billion in 2000 and was expected to increase at 13% annually. In 2008, the sales of the three top-selling single isomer drugs, Lipitor (atorvastation calcium), Plavix (clopidogrel bisulfate) and Nexium (esomeprazole magnesium) reached \$30 billion [11].

Chirality is also important in the agrochemical and food industry [12-15]. Enantiomers in agrochemicals can have diverse effects on plants and insects, and could bring out negative effects to the environment and be toxic to human health. In the food industry, a significant number of additives, flavors, fragrances and

fumigants, preservatives, growth regulators, pesticides and herbicides are chiral molecules, so are many organic components of foods.

1.1.2 Enantiomeric separation and principles

Generally, two strategies can be considered for the purpose of obtaining optically pure materials: direct enantioselective syntheses (asymmetry synthesis) and enantioseparation of racemic mixtures. Ideally, the desired pure enantiomer could be obtained by direct asymmetry synthesis without further treatment [16-21]. However this approach is not always efficient or cost effective. Several factors should be considered including catalyst efficiency, availability of starting materials, reaction conditions and kinetics, etc. Furthermore, there are not enough general catalysts available and a successful asymmetric synthesis procedure sometimes only affords one enantiomer.

Most chiral drugs are generated in the lab with equal amounts of enantiomers in an achiral environment. In order to get pure enantiomers, the separation of an enantiomeric mixture is necessary. Because of the same chemical and physical properties of the enantiomers, enantioseparation has been considered as one of the most difficult challenges in chemistry either from an analytical or a preparative viewpoint. The first example of optical resolution was reported by Louis Pasteur in 1850's. He made great success in hand-picking the individual crystals of tartaric acid by crystallization of the diastereomeric salts [22]. Derivatization is another important separation approach, in which optically pure reagents in pairs of

diastereomers are derivatized by some chiral resolving agents and the compound mixture can be separated by conventional techniques in physical chemistry [22]. During a long period of time, a substantive number of compounds have been resolved, mainly by fractional crystallization and derivatization. Other approaches used to obtain chiral molecules include enantiomeric enrichment, kinetic resolution, enzymatic resolution and partition in heterogeneous solvent mixtures [23]. Amongst these methods, crystallization and kinetic resolution are useful for preparative scale and are commonly employed for industrial processes, either as a single mean or in combination with chromatographic techniques [24].

During the past decades, the significance of enantiomeric separation for analytical scale application emerges rapidly in the arena of food safety, environmental analyses, agrochemical and drug industries [23-26]. In the asymmetry synthesis process or industrial preparative scale of enantiomers, enantioseparation methods on an analytical scale is important for determining enantiomeric purity and pharmacokinetic studies. Chiral analytical methods would include nuclear magnetic resonance (NMR), calorimetry, enzymes techniques, and polarimetry [27]. Chromatographic approaches are rapidly becoming one of the most commonly used enantioseparation approaches in both analytical and preparative scale.

The understanding of molecular interactions responsible for chiral recognition is of primary importance. Different views of separation principles are proposed [28]. The most acceptable one is the three-point interacting model (Figure 1-2),

proposed by Dalglish. It postulates that, for enantiomeric separation, three interactions are involved and at least one of them has to be stereoselective [29].

Figure 1-2 Three-point interaction model

As illustrated in Figure 1-2, there exist three interactions A—A', C—C' and D—D' between the chiral selector and enantiomer (I) while only two interactions A—A' and C—C' are possible between the selector and enantiomer (II). The discrimination effect of the two enantiomers falls on the interaction of D—D', the result is the different elution order of the two enantiomers. In conventional chromatography techniques, enantiomer (I) is eluted faster when this interaction is repulsive while retained longer when it is attractive. In capillary electrophoresis, the order is often more complicated due to the application of chiral additives in the mobile phase.

The interactions contributing to the asymmetric recognition process include hydrogen bonding, π – π interactions, ionic interactions, dipole-dipole interactions, inclusion complexation, metal complexation, hydrophobic interactions and so on [30-43]. Hydrogen bonding, an important interaction mode in chiral recognition, usually happens among amides and hydroxyl groups. In some cases, formation of multiple hydrogen bonds could afford complete resolution of the enantiomers. For

example, amide CSPs could resolve some derivatives of amino acids, hydroxyl acids and amino acids based on the formation of multiple hydrogen bonds [44]. As a large number of organic chiral drugs possess aromatic groups in their molecular skeletons, π - π association plays an essential role in enantiorecognition. The π -acceptor (electron acceptor) selectors are generally effective for resolving π -donor compounds through intermolecular π - π interaction while π -donor selectors are more powerful for separating π -acceptor enantiomers. Typical example is the (R)-N-(3,5-dinitrobenzoyl)phenylglycine phase introduced by Pirkle, having π -acceptor properties, showed chiral recognition ability to a broad spectrum of compounds with π -donor groups [45]. Similarly, for the ionic interaction effect, the charged selectors are more effective for resolving the racemates which are counter-charged. Lindner's group prepared cation exchange type CSPs using cinchona alkaloids as chiral selectors, which were successfully used in CEC [46]. The principle of ligand-exchange chromatography was introduced by Davankov and Rogozhin in the early seventies [47], when chiral recognition was carried out on the basis of formation of ternary mixed metal complexes between a chiral selector ligand and the analyte ligand. Another important interaction type is the inclusion complexation which is formed between the hydrophobic cavity of chiral selectors like cyclodextrins (CDs) and some functional groups of the analytes [48].

Enantioseparation depends on the combination of several interactions. It should also be noted that the "three-point" model does not always apply to all chiral

recognition cases. Sometimes the model is oversimplified or unreliable, especially for weakly bonded diastereomeric complexes like the inclusion complex between cyclodextrin (CD) host and chiral analytes since there is no well-defined binding orientation.

1.2 Modern-day enantiomeric separation techniques

In order to meet the rapid development of drug and food industries and the demand of protecting our environment, different analytical and preparative methods have been developed with the focus on chromatographic related methodologies (Figure 1-3) such as liquid chromatography (LC) [49], gas chromatography (GC) [50], supercritical fluid chromatography (SFC) [51], capillary electrophoresis (CE) and capillary electrochromatography (CEC) [52]. These methods each have their own advantages and disadvantages.

Figure 1-3 Modern-day enantiomeric separation techniques

Chiral LC includes chiral high performance liquid chromatography (HPLC) and

the more recent ultrahigh performance liquid chromatography (UHPLC) technique [53-61]. Chiral HPLC has dominated this area for several decades and is expected to continue to do so in many years to come due to its easy-handling and maturity. This technique is widely used for enantiomeric purity determination in asymmetry synthesis, pharmaceutical quality control, racemization process check and separation of enantiomers on a preparative scale. UHPLC is a fast developing technique on the basis of traditional LC by using silica beads with smaller size, reducing the column inner diameter and shortening the column length in order to improve the column efficiency. Although it has shown great promise in achiral separation, the feasibility and potential of UHPLC in the field of enantioseparation still needs further investigation.

Gas chromatography (GC) and supercritical fluid chromatography (SFC) are separation techniques with different carrier medium compared with LC. GC uses gas (generally nitrogen) while SFC employs carbon dioxide with some high polar organic solvent as the mobile phase [62-68]. GC is only used for chiral analysis whereas SFC can be used for preparative-scale separations because the carbon dioxide is relatively inexpensive, nontoxic, nonflammable and more environmentally friendly than the organic solvents typically used for LC.

CE is a kind of separation approach based on the differences in the charge density (charge-to-size ratio) of the analytes. Although the separation principle is quite different from that of chromatography, CE is sometimes classified into chromatographic separation techniques. As the “youngest” separation technique,

chiral CE has quickly garnered research interest and developed into one of the major separation techniques for analytical enantioseparations since the first CE enantio-recognition was reported in 1985 [69]. CE has the advantages of high peak efficiency and extremely low consumption of organic solvents over other chromatographic techniques. A large number of papers and books on CE have been published during the last decades [70-78]. CE methods are accepted by the regulatory authorities such as the FDA and the European Medicines Agency (EMA).

CE and LC possess different separation mechanisms (charge density in CE and lipophilicity in chromatography). Considering these characteristics, researchers combined CE and LC to introduce a new separation technique, capillary electrochromatography (CEC). CEC is a liquid phase analytical separation technique that is carried out with capillary columns by electroosmotically driven mobile phase at high electric field strengths in an apparatus similar to that used in capillary zone electrophoresis (CZE). As a hybrid of CE and HPLC, CEC combines the advantages of both techniques such as small sample quantity, accentuated separation factor and high efficiency [79-82]. Since the first enantioseparation on CEC was implemented, three CEC modes have been put into practice: open-tubular CEC (OT-CEC), packed-bed CEC (P-CEC) and monolithic CEC, each with its disadvantages and advantages. P-CEC is still a widely used CEC mode because of the easy conversion of conventional packing materials in HPLC to CEC. In P-CEC, the mobile phase is driven by electroosmotic flow (EOF)

generated from the electric double layer formed on the packed silica surface and capillary inner wall (Figure 1-4 left). The electro-driven mode forms a plug flow profile in the channels among the packed particles, rendering it much higher efficiency than the parabolic flow profile in the conventional LC (Figure 1-4 right) [79]. In addition, the small capillary allows for the analyses of much smaller quantity of samples than required in HPLC. This has resulted in widespread motivation in CEC enantioseparation.

Figure 1-4 Generation of EOF in P-CEC (left) and comparison of flow profiles in CEC and HPLC (right)

1.3 Chiral stationary phases

The simplest way to achieve enantioseparation is to add chiral additives directly into the mobile phase in the case of both HPLC and CE [49, 50]. This approach affords satisfactory separation results with simple operation in many cases. However, the chiral additives can normally be used only once and could not be regenerated after separations. In addition, the preparation of the chiral additives can be laborious and expensive. Moreover, some separation techniques cannot

allow for the employment of chiral additives, as in SFC and GC. A more practical approach is to use chiral stationary phases (CSPs) where the chiral selector is physically adsorbed or covalently bonded to silica or other supports. All the chromatographic enantiomeric separation techniques could employ CSPs. There are several types of CSPs applied in HPLC, GC, SFC and CEC, such as Pirkle-type CSPs [83, 84], polysaccharide-based CSPs [85-89], cyclodextrin CSPs [90-128], macrocyclic antibiotics CSPs [129, 130], chiral crown ether CSPs [131], protein CSPs and molecular imprinting-based CSPs [132] etc. Herein, this dissertation focuses on CD CSPs.

1.3.1 Cyclodextrin-based chiral stationary phases

1.3.1.1 Properties of cyclodextrins for enantioseparation

CDs have found important applications in areas such as biotechnology, asymmetry synthesis, asymmetry catalyst, achiral and enantioseparation, food industry, pharmaceutical and agricultural industries since they were first reported in late nineteenth century [90]. CDs (α , β , γ -CD) are non-reducing naturally occurring oligosaccharides consisting of D-glucopyranose units bonded through α -1,4 linkages (Figure 1-5). Hence, CDs are chiral structures with many chiral centers (for example, β -CD has 35 stereogenic centers). All the CD structures have a hydrophilic surface resulting from the 2-, 3- and 6-hydroxyl groups (6-OH are primary hydroxyl groups while 2- and 3-OH are secondary hydroxyl groups) on the rim. The nonbonding electrons on the glycosidic oxygen bridges in the α -1,4 bonds are oriented toward the center of the cavity, which gives the cavity some

Lewis-base character [91, 92]. These characteristics endow CD with a special capacity in accommodating a large variety of organic and inorganic compounds by inclusion complexation. This is the reason for their comprehensive application in chemistry and separation science.

According to a study by Armstrong [93], there should be some other requirements for chiral recognition by CDs other than the formation of inclusion complex. The chiral center or one substituent of the chiral center of an analyte must be near and interact with the hydroxyl groups of the CD rim to satisfy the requirement of the “three-point” model. As the stereoselective inclusion complexation and consequently the energy of interaction between the two enantiomers and CD rim –OH moieties could be different, it may result in the separation of the corresponding enantiomeric pair affording enantiodifferentiation.

Compared with the α - and γ -CDs, β -CD is more widely investigated in separation science. The reasons are not only due to their high chemical stability and low manufacturing cost but also on account of the special size of its hydrophobic cavity (cavity size: α -CD < β -CD < γ -CD) which affords the ability to form inclusion complex with numbers of organic and inorganic compounds [94].

Figure 1-5 Structures of CDs

1.3.1.2 Cyclodextrin as chiral stationary phases

The first application of CDs for enantioseparation was reported in 1959 in which CDs were employed as a selective precipitation/ crystallization agent for enantiomers [95]. From then on, CDs were studied either as mobile phase additives or stationary phases in chromatographic techniques [94].

CD derived stationary phases were originally designed for both enantiomeric separation and separation of structural and geometric isomers. Early studies of CD based stationary phases for enantioseparation focused on the polymerized and cross-linked gels composed of CDs which were not robust in chiral discrimination and often readily overloaded which resulting in distorted peaks [96]. Thereafter, researchers investigated the development of covalently bonded CD CSPs. In 1984, the first stable CD CSP (Cyclobond I) with high coverage of CD was developed by Armstrong *et al.* [97]. Subsequently, the CD derived CSPs were also commercialized by their group (Cyclobond 2000 series) and hundreds of chiral compounds have been resolved on these CSPs in different separation modes

(normal phase, reversed phase and polar organic mode) by HPLC [98-110].

The derivatization of the hydroxyl groups on the CD rims (secondary hydroxyl groups on the “upper” larger rim and primary hydroxyl groups on the “lower” smaller rim in Figure 1-5) could provide specific interactions with certain functional moieties and accommodate a variety of spatial requirements to produce highly selective separations for a versatile array of analytes. Up to now, the substitution groups have been restricted to alkyl, acetylated, hydroxypropylated, phenylcarbamoylated (naphthylethyl carbamoylated or 3,5-dimethylphenyl carbamoylated) (Figure 1-6).

Figure 1-6 Different substituent groups of CD CSPs

1.3.1.3 Interaction mechanism of the native and derived cyclodextrin CSPs

Inclusion complexation is the essential interaction in CD separation science. CDs could be regarded as “host” for “guest” molecules capable of penetrating the hydrophobic cavity and forming noncovalent “host-guest” complexes. Almost all CD applications involve complexation. A combination of other forces such as hydrogen bonding, hydrophobic interaction, electrostatic interaction, van der Waals interaction and dipole-dipole interaction generated from the different functional groups on the CD rim cooperatively governs the inclusion complexation behavior

of the CD “hosts” [97, 99, 101]. These interactions result in a selectivity-structure correlation, and this correlation forms the basis of enantiomeric separation using CD CSPs.

For native CD, hydrogen bonding accounts for the enantioseparation besides the inclusion complexation. It requires the existence of hydrogen bonding sites in the analyte structure. Preferred hydrogen bonding groups include carboxyl, carbonyl, amido, hydroxyl and amino moieties. After the “host-guest” inclusion is formed between the CD cavity and hydrophobic part of an analyte, such hydrogen bonding groups of the analyte on or near the chiral center must interact (attract or repulse) with the sterically fixed 2- or 3-hydroxyl groups of the CD cavity to allow for enantiorecognition [97, 105].

Methylation of the hydroxyl groups reduces the hydrogen bonding sites while enlarges the hydrophobic cavity and enhances the steric interactions on the secondary CD face. These CSPs exhibit good enantioselectivities to some specific solutes. Their chiral recognition is implemented through hydrophobic and steric interactions between the analytes and the methoxyl groups on the CD rim after inclusion formation [111, 112]. Since methylation could not introduce diverse effective interaction sites (like hydrogen bonding and π - π interaction sites), these CSPs are less effective towards a wide range of chiral compounds.

Hydroxypropylated CD CSPs have been considered as a very successful CSP. The pendant -OH groups of this CD derivative increase the flexibility of hydrogen

bonding and provide additional hydrogen bonding sites, therefore assisting in analyte immobilization and steric interactions especially for enantiomers with bulky substituents on the stereogenic center. Many chiral compounds that are partially resolved on native cyclodextrin CSP could undergo baseline resolution using similar separation conditions on these CSPs. Enhanced enantioseparation of some important drugs like conazoles, methadone, sertraline, Jacobsen's Catalyst and strigol can be achieved with these CSPs [104, 109]. However, the preparation process for these CSPs is relatively tedious and more costly.

Substituted phenyl or naphthylethyl carbamoylated CD CSPs have been labeled a multi-modal CSPs due to their various bonding sites. They could not only afford hydrogen bonding effects and dipole-dipole interactions but also hydrophobic and π - π interactions, and the different substitution groups on the aromatic rings can alter the nature of π - π interaction to make them more suitable for various racemates. Besides, the 6-hydroxyl groups could be replaced by ionic imidazole or pyridine groups to introduce ionic interactions [113-115].

1.3.1.4 Chemistry for bonding cyclodextrin derivatives

A successful CSP should not only show powerful chiral recognition ability but also present high stability under different separation conditions. Initial preparation of CD stationary phases only focused on cross-linking and physical coating, which were easy to handle but showed inherent deficiencies. For example, cross-linking CSP always induces distorted peak shapes and physical coating CSPs have low stability.

Some pioneering works on chemically bonding CDs were done by Fujimura *et al.* through amine and urethane linkages (shown in Figure 1-7 (i, ii)) [116, 117]. Meanwhile, amide linkages (Figure 1-7 iii) for CD immobilization were reported by Kawaguchi *et al.* [118]. All these linkages are unstable under broad separation conditions.

As mentioned in 1.3.1.2, the most successful CD CSPs were developed by Armstrong and coworkers using stable ether linkages (Figure 1-7 iv). This native CSP was obtained by reacting epoxy functionalized silica with CD using NaH in DMF. Direct functionalization of the native CD CSP afforded other functional CD CSPs. These non-selective approaches cannot readily achieve regioselective immobilization of CDs because of the randomly reacting sites of the CD rims and batch-to-batch reproducibility is poor.

Figure 1-7 Amine (i), urethane (ii), amide (iii) and epoxy ether linkages (iv)

Recently, a series of structurally well-defined CD CSPs were developed by our research group by immobilizing permethylated, peracetylated and perphenylcarbamoylated azido-CD derivatives through Staudinger reaction (Figure 1-8) [119, 120]. Some improved synthetic routes were also reported [121-125]. All the stationary phases possessed good stability and enantiorecognition ability to a large number of chiral compounds in normal and reversed phase mode HPLC. However, the Staudinger reaction was not successfully employed for the preparation of native CD CSPs because the hydroxyl groups on the rims could

induce side reactions with the precursor molecules.

Figure 1-8 Immobilizations of CDs by Staudinger reaction

Besides the above mentioned chemistry, there are also other linkages such as alkyl linkages (Chirasil-Dex) and thioether linkages (Chiradex-silica) (Figure 1-9). The Chiradex-silica shows very good chiral discrimination ability to mephobarbital in CEC and has been commercialized by Merck [126-128].

Figure 1-9 Structure of Chiradex-silica

It is interesting that the preparation of a novel native β -CD CSP based on “click” chemistry was attempted by Liang’s group using CuSO_4 and sodium

ascorbate as the catalyst in 2008 [133]. However, the purification process using this method was complicated due to the formation of copper particles and the amount of CD immobilized was very low. Anyway, this work provided good insights to the development of novel CD CSPs.

1.4 “Click” chemistry

“Click” chemistry [134], coined by Sharpless and coworkers in 2001, refers to chemical reactions that are modular, versatile, regiospecific, simple to operate and high yielding. They should be conducted using readily available reactants and should avoid chromatographic purifications. Developed by Sharpless and coworkers in 2002, the Cu (I) catalytic 1,3-dipolar cycloadditions of terminal alkynyl and azido for the formation of 1,2,3-triazolyls (Figure 1-10) fulfill all these requirements [135].

The original reaction is Huisgen cycloaddition which affords 1,4- and 1,5-triazolyl regioisomers by thermal treatment of the reactants. By introducing Cu (I) catalyst, this cycloaddition is governed by kinetic control and becomes highly reliable and stereoselective. 1,4-triazolyl products are exclusively obtained with high yields by this approach. The installations of terminal azido and alkynyl groups to molecular skeleton are fairly facile and quite stable under standard conditions. More importantly, “click” chemistry is inert towards various functional groups, solvents and not sensitive to water and air, the latter of which is usually avoided for a large number of reactions. Furthermore, this reaction makes full use

of the azido group which usually exists as an intermediate of amino group. All these characteristics make this reaction “almost perfect” [135]. As a result, in many recent publications, “click” chemistry is commonly considered as Cu (I) catalytic 1,3-dipolar cycloaddition reaction.

Figure 1-10 Cu(I) catalytic 1,3-dipolar cycloaddition

The mechanism (Figure 1-11) of “click” chemistry was first proposed by Sharpless and coworkers [135], modified by Finn *et al.* [136] and finally revised by Bock *et al.* [137]. This reaction has been proven to proceed via a stepwise catalytic process which could reduce the activation barrier. Firstly, the Cu (I) complex is readily anchored onto the alkynyls via Sonogashira coupling followed by the terminal hydrogen deprotonation to produce the Cu (I)–alkynyl ligand. Thereafter, N^1 replaces one ligand to form a new complex and meanwhile makes the azido more nucleophilic. The result is that N^3 attacks the C^4 forming a ring which undergoes several rearrangements and thereby translates to the Cu (I)–triazolyl complex. The final triazolyl product is obtained after the protonation step, and at the same time, the catalyst is regenerated.

Although the most commonly used catalytic system for “click” chemistry was developed by in situ-generation of Cu (I) through reduction of Cu^{2+} with sodium ascorbate in aqueous alcoholic solutions [135], many other types of catalysts have

also been described to work well such as CuI, CuBr, CuOTf·C₆H₆ and [Cu(NCCH₃)₄][PF₆] [138-139]. Even copper wire was found to be effective to this reaction. Some kinds of Cu (I) modified zeolites were also reported to be usable for the “click” reaction [140].

Figure 1-11 Mechanism of “click” chemistry

“Click” chemistry has found numerous applications in many scientific areas. Over ten reviews describe its applications from different perspectives [141-151]. They include drug discovery, bioconjugation, polymer and materials science, and drug delivery.

1.4.1 “Click” chemistry in drug discovery and bioconjugation

“Click” chemistry complements and extends the present methods for biomedical research and modern day medicinal chemistry. The development of “click”

chemistry affords novel approaches to screen drug libraries. According to the review by Sharpless [151], the application of the “click” chemistry in drug discovery approaches have been realized in three aspects: (i) Synthesis of neoglycoconjugates. Gonzalez and coworkers synthesized a series of multivalent triazole-bridged neoglycoconjugates via “click” chemistry with high yields and complete regioselective control [152]. It was also found that microwave irradiation could greatly accelerate the reaction, even heptakisazido- β -CD could be converted to their triazolyl derivatives; (ii) Preparation of fucosyltransferase inhibitors. A typical case is the identification of nanomolar inhibitors which were fabricated by “click” chemistry combination of azides and a guanosine diphosphate (GDP) derived alkyne [153]; (iii) Development of HIV protease inhibitors. A strategy for curing AIDS is to find new inhibitors of HIV protease to destroy or delay the final stage of virus maturation. Some novel inhibitors based on hydroxyethylamine peptide isosteres have been prepared via “click” route and proven to be effective against the HIV protease [154].

Bioconjugation techniques involve covalent immobilization of synthesized functional molecules to biomolecular templates. Examples include the functionalization of proteins and nucleic acids with chelates, radioisotopes, fluorophores and ligands; conjugation of proteins; and linkage of carbohydrates to peptides. However, only a small amount of reactions were proved to be useful in this area. The emergence of “click” chemistry affords a powerful strategy to meet the demand of *in-vivo* bioconjugation. The first bioconjugation via “click”

chemistry was hinted in Meldal's study which coincidentally engendered the first peptidotriazolyis by solid phase "click" reaction [155]. With the discovery of "click" chemistry in water and organic solvents, facile introduction of various appendages onto a broad range of biomolecules such as peptide, oligosaccharides, glycoconjugates and DNA has been achieved. Labeling intact Cowpea mosaic virus (CPMV) with fluorescein was successfully performed using "click" chemistry by Finn *et al.* [156]. Cravatt and coworkers proved the effectiveness of this reaction in the field of activity-based protein profiling (ABPP) [157]. An example of effective DNA labeling was shown in Ju's study wherein primers for the Sanger dideoxy chain termination reaction for DNA sequencing was successfully prepared via "click" chemistry [158].

1.4.2 "Click" chemistry on silica materials

Surface modification of materials requires very stable bridges to link functional molecules to different kinds of supports such as various silica materials, polymers, metal carriers, carbon materials like C60 and carbon nanotubes [141-144]. Silica, being inexpensive, stable, easy-obtaining and having simple functionalization chemistry, has been broadly used in chromatography, microchip, membrane, industrial catalyst, drug delivery as well as biological sciences [145-151]. "Click" chemistry has been investigated in silica surface modification area.

The first case of silica surface modification by "click" chemistry was done by Hoffmann *et al.* by immobilizing substituted acetylenes to powdered silica and

silicon wafers in 2004 [159]. Subsequently different kinds of functional molecules were anchored to silica via “click” chemistry, most of which were carried out by immobilizing alkynyl derived compounds to azido modified silica. Besides, several kinds of “clickable” silica materials have been reported like “clickable” SBA-15, which is prepared by an one-pot co-condensation. This material embodies abundant azido groups inside its cylindrical pores and could potentially become a good platform for the development of covalently bonded sensors and catalysts [160-166]. Some representative immobilizations on silica surfaces via “click” chemistry are depicted in Figure 1-12.

1.4.3 “Click” chemistry on nanomaterials and polymers

“Click” chemistry provides a reliable way for the functionalization of various nanomaterials including silica, TiO₂, gold and silver, magnetic and polymer nanoparticles [167-172]. The functional molecules include simple compounds to biomolecules. In addition, functionalization of polymers process represents an essential step in polymer science. Linking numerous ligands onto polymers and dendrimers demands efficient and reliable coupling reactions. The limited solubility of many polymers requires the reaction to take place in heterogeneous media. The “click” chemistry approach affords an important contribution in this aspect [173, 174]. Several reviews have highlighted the original literature papers which demonstrated the wide applications of “click” chemistry in the field of polymer sciences [144-146, 149].

Click functionalized silica for achiral separation

Click Cinchona alkaloids on silica for enantioseparation

Click tryptsin to mesoporous SBA-15

Click organosilane groups to mesoporous silica materials

Click monolayers on silica surfaces

Click Pd and Pt Pincer organometallics on silica

Figure 1-12 “Click” chemistry on silica surfaces. Ref [159-166]

1.5 Research objectives and scope

As reviewed in section 1.4, “click” chemistry has become one of the most robust tools for surface grafting of a wide variety of functional molecules. This motivates us to develop different novel CSPs based on this approach. CDs are chosen as chiral selectors because of their strong enantiorecognition potential to many racemic compounds. As the emerging capillary electrochromatography technique offers distinct advantages, one part of this dissertation will focus on the fabrication of the packed-bed capillaries with the newly prepared CD CSPs and their applications on CEC enantioseparation. Furthermore, the enantiorecognition abilities of the novel CD CSPs will be fully investigated by separating a large number of racemic compounds using the more common and versatile HPLC technique.

The research objectives of this dissertation are:

- i. To synthesize different azido CD derivatives.
- ii. To develop a facile and reliable approach to immobilize the CD derivatives onto silica surfaces via “click” chemistry.
- iii. To fabricate capillary columns with the desired CSPs and apply them in capillary electrochromatography for enantioseparation.
- iv. To investigate the enantiorecognition abilities of the “click” chemistry derived CSPs in HPLC for a broader range of chiral compounds such as aryl alcohols, flavonoids, β -blockers and dansyl amino acids, etc.

Chapter 2*

Syntheses of cyclodextrin derivatives and preparation of cyclodextrin CSPs *via* “click” chemistry

* Much of this chapter has been published (*Tetrahedron Lett.* 2008, 49, 5190), permission has been attached.

2.1 Synthetic design and methodologies

One of the main objectives of this project is to develop a facile and reliable synthetic approach for the preparation of cyclodextrin CSPs based on “click” chemistry. Scheme 2-1 outlines synthetic route used. As described in Chapter 1, “click” chemistry is a copper (I) catalyzed triazole formation reaction between terminal alkynyl and azido moieties. Accordingly, terminal alkynyl or azido groups should be introduced to the cyclodextrin skeleton and silica surface in order to achieve “click” immobilization. Two strategies have been employed to afford five novel CSPs. One route depicts the formation of the desired **CSP1-3** where β -cyclodextrin was first selectively tosylated at one 6-OH position followed by azidolysis reaction to afford mono(6^A-azido-6^A-deoxy)- β -CD **3** (N_3 - β -CD) which was subsequently perfunctionalized to afford perphenylcarbamoylated and permethylated mono(6^A-azido-6^A-deoxy)- β -CD derivatives ready for “click”. In the other synthetic route, selective iodination of β -CD was performed first at all the 6-OH positions to afford product **5** which thereafter reacted with sodium azide to afford heptakis(6-azido-6-deoxy)- β -CD (heptakis- N_3 - β -CD) **6**. Phenylcarbamoylation of **6** afforded heptakis(6-azido-6-deoxy)-2,3-di-O-phenylcarbamoylated- β -CD (heptakis- N_3 -Ph-CD) **7** which was ready for “click”.

The introduction of alkynyl groups onto the silica surfaces followed a two-step approach with propiolic acid and 3-aminopropyltriethoxysilane as the starting materials.

Thereafter, the desired CSPs were prepared by immobilizing the azido- β -CD derivatives **3**, **4a**, **4b**, **6** and **7** onto the alkynyl modified silica **10** in the presence of CuI(PPh₃) **9** soluble in organic solvents.

Scheme 2-1 Reagents and conditions: (a) imidazole/ CH_2Cl_2 and $\text{H}_2\text{O}/\text{rt}/\text{TEA}$; (b) $\beta\text{-CD}/\text{H}_2\text{O}/\text{rt}$; (c) $\text{NaN}_3/\text{DMF}/\text{rt}$; (d) **4a**: $\text{CH}_3\text{I}/\text{DMF}/\text{NaH}/\text{rt}$ and **4b**: $\text{PhN}=\text{C}=\text{O}/\text{pyridine}/85^\circ\text{C}$; (e) propiolic acid/ $\text{DCC}/\text{CH}_3\text{Cl}/\text{rt}$; (f) silica/toluene/reflux; (g) iodine/ $\text{DMF}/\text{PPh}_3/90^\circ\text{C}$; (h) $\text{NaN}_3/\text{DMF}/90^\circ\text{C}$; (i) $\text{PhN}=\text{C}=\text{O}/\text{pyridine}/90^\circ\text{C}$; (j) $\text{DMF}/\text{CuI}(\text{PPh}_3)/80^\circ\text{C}$

2.2 Preparation of CD derivatives

2.2.1 Syntheses and characterization of monoazido-CD derivatives

Syntheses. The preparation of monoazido-CD derivatives started from the synthesis of an important electrophilic reagent 1-(*p*-tosyl)-imidazole **1** in a simple way with high yield as described in Chapter 6. The synthesis of **1** was conducted by nucleophilic substitutions between imidazole and *p*-toluenesulfonyl chloride with dichloromethane and water as solvents using triethylamine as the phase transfer catalyst. The combined organic layer was dried on anhydrous MgSO₄, concentrated and added hexane. Pure product was thereafter crystallized out from the concentrated dichloromethane solution. The yield of **1** in this approach reached 95%, higher than the existing method (89%), in which the reaction was conducted in homogenous solution using anhydrous dichloromethane as the solvent [175]. Selective tosylation at 6-OH position was then affected, albeit in a yield of 30%, by reacting electrophilic 1-(*p*-tosyl)-imidazole with the most nucleophilic primary hydroxyl groups of β -CD according to a literature report [175]. TsO- β -CD **2** is an important CD intermediate for the easy introduction of various functional groups such as halides, azide, amine and imidazole groups. One of the key intermediates, mono(6^A-azido-6^A-deoxy)- β -CD **3** (N₃- β -CD) for “click” chemistry, was obtained by treating **2** with excess sodium azide in water followed by complexation with 1,1,2,2-tetrachlorethane and subsequent decomposition of the complex in boiling water. According to the procedures described in the previous report [119], permethylation of **3** was affected using sodium hydride and iodomethane in

anhydrous DMF followed by flash chromatography affording mono(6^A-azido-6^A-deoxy)-permethylated- β -CD **4a** (Me-N₃-CD). The perphenylcarbamoylation reaction was carried out in anhydrous pyridine (dried over calcium hydroxide for 4 h) with excess phenyl isocyanate (~1:60 molar ratio) and the crude product was purified by flash chromatography giving mono(6^A-azido-6^A-deoxy)- perphenylcarbamoylated- β -CD **4b** (Ph-N₃-CD) in a yield of 49%.

Characterization. The products **2**, **3**, **4a** and **4b** were characterized by FTIR, NMR, MS and elemental analysis. The FTIR spectra collected as KBr pellets for compound **2**, **3**, **4a** and **4b** are shown in Figure 2-1.

As observed, compounds **3**, **4a** and **4b** all exhibit absorptions at 2107 cm⁻¹ due to the N-N stretching vibration of azido group after the azidolysis reaction on compound **2**. In the spectra of **4a** and **4b**, the strong broad absorption at 3400 cm⁻¹ for hydroxyl groups disappears indicative of successful functionalization of all the hydroxyl groups. For N₃-Me-CD **4a**, the strong absorptions between 2800 and 3000 cm⁻¹ are attributed to the enhanced vibration of C-H on the methyl groups. For N₃-Ph-CD **4b**, the strong absorption of C=O appears at 1739 cm⁻¹ and the peaks at 1602 and 1500 cm⁻¹ are the signals accounting for the aromatic rings.

Figure 2-1 FTIR spectra of **2**, **3**, **4a** and **4b**

^1H NMR resonances at δ 7.73, 7.42 (H-Aromatic) and 2.50 (CH_3) ppm of compound **2** disappeared in the ^1H NMR spectrum of **3**, indicating the complete replacement of tosyl group by $-\text{N}_3$. Successful permethylation could be reflected by the disappearance of resonances at δ 4.54 and 5.62 ppm ascribing to the $-\text{OH}$ of the CD with the concomitant growth of new signals around 3.50 ppm for the protons of $-\text{OCH}_3$ groups. For compound **4b**, resonances between 6.88 and 7.45 ppm indicate the entry of phenylcarbamoyl moieties. The MS data further prove that the desired products **3**, **4a** and **4b** have been obtained. MS (m/z) for **3**: 1182.5 (found) and 1182 $[\text{M}+\text{Na}]^+$ (calculated); for **4a**: 1311.2 (found) and 1311.2 $[\text{M}+\text{H}]^+$ (calculated); for **4b**: 3676.2 (found) and 3676.1 $[\text{M}+\text{K}]^+$ (calculated).

2.2.2 Syntheses and characterization of heptakisazido-CD derivatives

Syntheses. The preparation of heptakis(6-azido-6-deoxy)-2,3-di-O- β -CD (heptakis N_3 -CD) **6** followed a two-step strategy according to the procedure described in the literature [120]. β -CD was firstly treated with iodine in anhydrous DMF with triphenylphosphine as catalyst where all the primary hydroxyl groups were replaced by iodide to afford compound **5**. Thereafter, the iodide groups on 6-OH positions were converted to azido groups in DMF. Phenylcarbamoylation of the residual hydroxyl groups on **6** was achieved in anhydrous pyridine with excess phenyl isocyanate to afford heptakis(6-azido-6-deoxy)-2,3-di-O-phenylcarbamoylated- β -CD (heptakis- N_3 -Ph-CD **7**).

Characterization. FTIR spectra (KBr) (Figure 2-2) show the typical absorptions of product **6** and **7** at 3400, 3317, 2104, 1739, 1600 and 1500 cm^{-1} . Compared with **3** and **4b**, the absorption at 2104 cm^{-1} for **6** and **7** is much stronger than **4a** and **4b** on account of the accentuated absorption of the seven azido groups located on the primary CD rims. ^1H NMR spectrum of **7** shows typical signals between 6.8 and 7.5 ppm corresponding to the protons of phenylcarbamoyl groups. MS of the product **7** show a peak at m/z 2997.3 $[\text{M}+\text{Na}]^+$, indicating a molecular formula of $\text{C}_{187}\text{H}_{175}\text{N}_{23}\text{O}_{56}$. The characterization data of **6** and **7** are consistency with that reported in the literature [120].

Figure 2-2 FTIR spectra of **6** and **7**

2.2.3 Investigation of “click” chemistry on azido-CD derivatives

Before the “click” chemistry immobilization onto silica surfaces, the Cu (I) catalytic 1,3-Huisgen cycloaddition (“click” chemistry) was first investigated by derivatization of monoazido-CD derivatives with two electron-deficient reagents (propionic acid and ethyl propiolate) using the well-known cuprous sulfate and sodium ascorbate catalytic system. Dimethylformamide (DMF) was chosen as the organic solvent in order to solubilize all the CD derivatives. The reactions are depicted in Scheme 2-2.

Scheme 2-2 “Click” chemistry on CD derivatives

The FTIR spectrum of the resulting residue for the first reaction was found to take on an absorption at 2107 cm^{-1} , which indicated the existence of the azido group. ^1H NMR results did not exhibit the representative signal for the triazolyl hydrogen around δ 8.5 ppm. The MS showed a representative peak of **3**. This indicates that treatment of N_3 - β -CD with propionic acid did not lead to the formation of triazolyl with the experimental catalytic system. The reason might be

due to the interference of carboxyl group on the complexation between the alkynyl group and Cu (I) catalyst.

However, the cycloaddition reaction could be readily implemented by replacing propiolic acid with ethyl propiolate. N₃-β-CD and ethyl propiolate were dissolved in DMF at a molar ratio of 1:1 with the addition of anhydrous copper sulfate. After dropwise adding of the sodium ascorbate solution, the color of the reaction system changed to light yellow and a brown precipitate were formed within a few hours, which suggested the reduction of Cu²⁺ to Cu⁰ under the reaction conditions. The product 1-(6^A-deoxy-β-CD)-4-(ethoxycarbonyl)-1,2,3-triazole (ETCD) **11** was characterized by FTIR, ¹H NMR, and ESI-MS. Direct evidence for the formation of triazolyl linkage was provided by ¹H NMR spectra (Figure 2-3 shows the ¹H NMR of N₃-β-CD and ETCD **11**). Compared with N₃-β-CD, the new signal at δ = 8.7 ppm in the ¹H NMR spectrum of **11** is assigned to the triazolyl proton which demonstrates the formation of 1,4-regioisomer exclusively, ethoxyl resonances are located at 4.3 ppm (CH₃CH₂O) and 1.3 ppm (CH₃CH₂O-).

The other two products 1-(6^A-deoxypermethylated-β-CD)-4-(ethoxycarbonyl)-1,2,3-triazole (ETMeCD) **12** and 1-(6^A-deoxyperphenylcarbamoyle-β-CD)-4-(ethoxycarbonyl)-1,2,3-triazole (ETPhCD) **13** were synthesized under the same reaction condition and purified by flash chromatography. ¹H NMR of **12** and **13** also presented the signals of triazolyl proton and methyl proton (Chapter 6) which indicates the generation of triazolyl ring.

Figure 2-3 ^1H NMR spectra of $\text{N}_3\text{-}\beta\text{-CD}$ **3** and ETCD **11**. (a) ETCD (b) $\text{N}_3\text{-}\beta\text{-CD}$

The FTIR spectra (KBr) of **11**, **12** and **13** are shown in Figure 2-4. The typical absorption of azido groups at 2107 cm^{-1} for the three $\text{N}_3\text{-}\beta\text{-CD}$ derivatives (Figure 2-1) have completely disappeared with the concomitant growth of new absorptions centred around 1740 cm^{-1} for **11** and **12** indicated the existence of newly introduced carbonyl group. In addition, the ESI-MS showed M^+ peaks ($[\text{M}+\text{Na}]^+$ 1280.4 for **11**, 1560.4 for **12** and 3657.8 for **13**) corresponding to the expected compounds (Chapter 6). These data combined with the ^1H NMR results confirmed the successful syntheses of **11**, **12** and **13** through a 1,2,3-triazolyl linkage.

Figure 2-4 FTIR spectra of **11**, **12** and **13**

2.3 Preparation of the CD-based CSPs via “click” chemistry

2.3.1 Preparation of alkynyl modified silica

Encouraged by the forgoing model compounds study, we proceeded to effect transfer of the reaction scheme to the CSP preparation protocol. A necessary step was to introduce an alkynyl group onto the silica surface. As 3-aminopropyltriethoxysilane is one of the most commonly used coupling agent, it was anticipated to react with propiolic acid to afford the prerequisite terminal alkynyl moiety. In case of the hydrolysis of 3-aminopropyltriethoxysilane, the reaction was required to be carried out under an anhydrous condition. Since this is a dehydration reaction, the water produced during the reaction needed rapid removal. Alternatively, a protocol without water formation would be favorable. Considering all the requirements, we chose anhydrous dichloromethane as the reaction solvent and dicyclohexylcarbodiimide (DCC) as the dehydrating agent.

The reaction was carried out with the initial formation of an active ester between propiolic acid and DCC followed by reacting with 3-aminopropyltriethoxysilane to afford N-3-(triethoxysilyl)propyl-2-propynamide (Scheme 2-3).

Scheme 2-3 Synthesis of N-3-(triethoxysilyl)propyl-2-propynamide **8**.

Upon the completion of reaction (within one hour), the dicyclohexylurea formed was filtrated off and the minor excess of propiolic acid was removed by co-evaporation with dry toluene to afford **8** as light yellow oil. The FTIR of product **8** depicted a fairly strong absorption at 2110 cm^{-1} ascribable to the terminal alkyne. In the ^1H NMR, characteristic peak for the alkyne moiety can also be seen at the singlet signal at δ 2.28 ppm. MS of this product showed a peak at m/z 296.1 $[\text{M}+\text{Na}]^+$, indicating a molecular formula of $\text{C}_{12}\text{H}_{23}\text{NO}_4\text{Si}$. The synthesized alkynyl coupling agent **8** was thereafter immobilized onto $5\text{ }\mu\text{m}$ silica (dried at 160°C under vacuum for 24 h) in anhydrous toluene following an established protocol [121, 122]. Unreacted impurities in the crude product were removed by rinsing with acetone in Soxlet extractor.

In the FTIR spectrum (KBr) (Figure 2-5) of the alkynyl modified silica **10**, a new absorption at 2121 cm^{-1} is evident, and elemental analysis result showed a carbon content of 5.32% for **10**. The analytical results indicate that the alkynyl groups have been successfully linked onto the silica surface.

Figure 2-5 FTIR of bare silica and alkynyl silica **10**

2.3.2 Preparation of Cu (I) catalyst

With the alkynyl modified silica and terminal azido CDs in hand, the surface immobilization via “click” chemistry approach was first tried with cuprous sulfate and sodium ascorbate catalytic system in DMF. However, a large amount of brown Cu (0) particles were formed during the reaction which made purification of the resultant product quite difficult. To solve this problem, a Cu (I) catalyst soluble in the reaction solvent was proposed, which could not only improve the catalytic effect but also simplify the purification procedure by just extracting the crude product with acetone and methanol to remove all the undesired organic impurities.

Direct use of catalyst CuI and ACN as solvent was a simple way for “click” as CuI could be dissolved in ACN. However, this was not suitable in current study because of the low solubility of N₃-β-CD in ACN.

Herein, an organic soluble catalyst cuprous iodide-triphenylphosphine complex [CuI(PPh₃)] was prepared by treating CuI with triphenylphosphine in ACN (Scheme 2-4). Its structure was characterized by FTIR (KBr) and elemental analysis (Chapter 6). Employing this catalyst, the formation of Cu (0) particles was avoided and purification of the CSP was simplified.

Scheme 2-4 Preparation of cuprous iodide-triphenylphosphine complex

2.3.3 Preparation of CSP1-3 via “click” chemistry

The “click” immobilization of N₃-β-CD, N₃-Me-CD and N₃-Ph-CD onto alkynyl modified silica was readily achieved in DMF via adding 10 mmol% of the synthesized organic soluble CuI(PPh₃) and the reaction system was heated to 80 °C for 2 days. It is noteworthy that vigorous stirring was necessary to properly disperse the silica beads, hence stirring speed > 500 rpm was required. Three different CD based CSPs have been obtained (Scheme 2-5).

Scheme 2-5 Preparation of the **CSP1**, **CSP2** and **CSP3**

The mechanism of the “click” chemistry has been illustrated in Chapter 1. Crude products obtained by filtration were washed twice with DMF and followed by Soxhlet extraction with acetone and methanol for 24 h to remove the organic impurities.

The functional groups of the prepared **CSP1-3** were characterized by FTIR (KBr) (Figure 2-6). As shown in Figure 2-6, the absorption at 2121 cm⁻¹ ascribed to the alkynyl group was greatly diminished (nearly invisible) after the “click” reaction. Instead, there appeared new absorptions at 2943 cm⁻¹ (due to methylene on the glucose units) for **CSP1**, 2800~3000 cm⁻¹ (corresponds to the methyl groups) for **CSP2** and 1724, 1545, 1448 cm⁻¹ (corresponds to carboxyl groups and phenyl rings) for **CSP3**.

Figure 2-6 FTIR of **CSP1**, **CSP2** and **CSP3**

The elemental analyses data for **CSP1-3** (Table 2-1) show that **CSP1** afforded the highest surface loading at $0.59 \mu\text{mol}\cdot\text{m}^{-2}$, while **CSP3** contained least CD molecules. Since “click” chemistry is a ring forming reaction with Cu (I) catalyst, the steric effects may influence the reaction process. For $\text{N}_3\text{-Ph-CD}$, the azido group is surrounded by several phenylcarbamoyl groups which take on a large steric volume. This reduces the chance for the azido group to form complex with the catalyst hence difficult to form the triazolyl bridge.

Table 2-1 Elemental analyses of **CSP1-3**

Bonded Silica	Elemental analysis (%)			Surface loading ($\mu\text{mol}\cdot\text{m}^{-2}$)
	C	H	N	
Alkynyl silica 10	5.320	0.986	0.846	-
CSP1	14.35	2.639	1.490	0.59
CSP2	16.62	2.616	1.630	0.51
CSP3	12.49	1.794	1.976	0.11

Surface loading = $\{C \% / (12 \times N_c \times S_{\text{silica}})\} \times 1000$, where *C %* is derived from elemental analysis of carbon; N_c is the carbon atom number per CD molecule and S_{silica} is the surface area of silica gel.

The FTIR and elemental analyses data verified that the **CSP1-3** have been successfully prepared via “click” chemistry.

2.3.4 Preparation of **CSP4** and **CSP5** via multiple “click” chemistry

Two more CSPs (**CSP4** and **CSP5**) were prepared by linking heptakis- $\text{N}_3\text{-CD}$ **6** and heptakis $\text{N}_3\text{-Ph-CD}$ **7** to the alkynyl modified silica via multiple “click” chemistry. The desired CSPs embodied multiple triazolyl linkages which may

enhance their stability over the ones with single linkage, especially in acidic and basic media. In addition, analytes could only enter the CD cavity from the secondary CD face due to the steric hindrance of triazolyl groups on the primary side. This gives us a good way to evaluate how the two CD rims and CD hydroxyl groups work in the complex formation process by comparing the separation results with those on **CSP1** and **CSP3**.

Scheme 2-6 Preparation of **CSP4** and **CSP5**

Scheme 2-6 depicts the multiple "click" immobilization and the structures of **CSP4** and **CSP5**. The multiple "click" chemistry reaction was carried out at 80 °C for two days by reacting the heptakis N₃-CD derivatives with the alkynyl functionalized silica in DMF under the existence of CuI(PPh₃). Successful immobilization is affirmed by the FTIR (KBr) (Figure 2-7) and elemental analyses data (Table 2-2).

Figure 2-7 FTIR of **CSP4** and **CSP5**

The distinct absorption band at 2121 cm^{-1} attributable to the alkynyl modified silica was greatly weakened after the multiple “click” reaction. There were no obvious absorptions at 2107 cm^{-1} in the spectrum of **CSP4**, indicating that most of the azido groups have been converted to triazolyl linkages. The extremely weak absorption at about 2107 cm^{-1} of **CSP5** might be caused by the few unreacted CD azido groups. More importantly, there appeared representative absorptions corresponding to the synthesized CD derivatives (2943 cm^{-1} for C-H of **CSP4** and

1738 cm⁻¹ for C=O of **CSP5**). Elemental analyses showed the great increase of carbon and nitrogen contents compared with alkynyl silica. The surface loading reached 0.35 and 0.10 μmol·m⁻² for **CSP4** and **CSP5**, respectively.

Table 2-2 Elemental analysis of **CSP4** and **CSP5**

Bonded Silica	Elemental analysis (%)			Surface loading (μmol·m ⁻²)
	C	H	N	
Alkynyl silica 8	5.94	1.18	1.00	-
CSP4	11.31	2.26	3.90	0.35
CSP5	13.57	1.89	3.18	0.10

Surface loading = {*C* % / (12 × *N_c* × *S_{silica}*)} × 1000, where *C* % is derived from elemental analysis of carbon; *N_c* is the carbon atom number per CD molecule and *S_{silica}* is the surface area of silica gel.

2.4 Summary

Different azido-β-CD derivatives (N₃-β-CD, N₃-Me-CD, N₃-Ph-CD, heptakis-N₃-β-CD and heptakis-N₃-Ph-CD) have been synthesized. The azido group on the CD ring afforded facile reaction with ethyl propiolate via the formation of triazolyl linkage with copper sulphate and sodium ascorbate as the catalytic system (“click” chemistry). Three novel CD derivatives (ETCD **11**, ETMeCD **12** and ETPhCD **13**) have been derived in this way from N₃-β-CD, N₃-Me-CD and N₃-Ph-CD. However, the same reaction could not be effected between the azido-CD derivatives and propiolic acid under similar reaction conditions, which might be the result of interference of carboxylic acid group in the complexation between the alkynyl group and Cu (I) catalyst.

The “click” chemistry for the preparation of CD based CSPs was carried out by

anchoring the synthesized azido-CD derivatives to the alkynyl modified 5 μm silica catalyzed by $\text{CuI(PPh}_3\text{)}$ which was obtained from the reaction between cuprous iodide and triphenylphosphine in acetonitrile.

Three CSPs (**CSP1**, **CSP2** and **CSP3**) were prepared via single triazolyl linkages from $\text{N}_3\text{-}\beta\text{-CD}$, $\text{N}_3\text{-Me-CD}$, $\text{N}_3\text{-Ph-CD}$ with surface loading as 0.59, 0.51 and $0.11 \mu\text{mol}\cdot\text{m}^{-2}$, respectively. **CSP4** and **CSP5** were engendered via multiple triazolyl spacers from heptakis $\text{N}_3\text{-}\beta\text{-CD}$ and heptakis $\text{N}_3\text{-Ph-CD}$ with surface loading at 0.35 and $0.10 \mu\text{mol}\cdot\text{m}^{-2}$, respectively. The FTIR and microanalyses results confirmed the successful preparation of the five CSPs. Our result shows that “click” chemistry is a facile approach to the immobilization of cyclodextrins onto the silica surfaces.

Chapter 3*

Chiral capillary electrochromatography (CEC) with “click” chemistry derived cyclodextrin CSPs (CSP1-3)

* Much of this chapter has been published (*Electrophoresis* 2009, 30, 705), permission has been attached.

3.1 Introduction

Capillary electrochromatography (CEC) embodies a hybrid of features between HPLC and CE including small sample quantities, good selectivities and high efficiencies [52]. The first enantioseparation by open tubular CEC (OT-CEC) was described by Mayer and Schurig in 1992 [176]. They resolved enantiomers of 1,1'-binaphthyl-2,2'-diylhydrogenphosphate and 1-phenylethanol using open tubes wall-coated with Chirasil-Dex. Later, Li and Lloyd demonstrated the first enantioseparation using CEC with packed capillaries (P-CEC) [177]. Very recently, another CEC mode, monolith CEC has been developed [178]. Low phase ratios and poor loading capacity have made OT-CEC less popular for enantioseparation. P-CEC is a widely used CEC mode because the conventional chiral stationary phases used in HPLC can be easily employed for this technique. Reports of enantioseparation using P-CEC have focused on pressure-supported CEC in which the column is pressurized at the inlet end to supply additional eluting force to drive the mobile phase [179-181]. However, in this situation, high pressure can replace EOF as the main eluting force and mask the role of EOF in the enantioseparation process.

In Chapter 2, we have described “click” chemistry for coupling N_3 - β -CD derivatives and ethyl propiolate as model reactions for attaching CDs to an alkynyl functionalized silica surface. The successful development of this reaction allowed us to prepare three novel CSPs by immobilizing N_3 - β -CD, N_3 -Me-CD and N_3 -Ph-CD onto alkynyl functionalized silica to afford **CSP1**, **CSP2** and **CSP3**,

respectively.

In this chapter, we shall discuss the three new chiral capillaries which have been prepared from **CSP1**, **CSP2** and **CSP3**. The newly fabricated capillaries were evaluated for CEC enantioseparation of flavonoids and racemic aryl alcohols. The influences of CEC parameters such as the applied voltage, organic modifier content and buffer pH on the EOF and resolution were also investigated.

3.2 Capillary columns fabrication and CEC operation

Column packing becomes increasingly difficult with the reduction of the column inner diameter. The commonly used CEC capillary has inner diameter smaller than 100 μm . Fabrication of capillary columns is considered as an art. A successful packed capillary column should afford high efficiency, reproducibility, and stability. In this study, slurry method was applied for the capillary packing. The fabrication procedure is illustrated in Figure 3-1.

Figure 3-1 Fabrication of the capillary column

As shown in Figure 3-1, the fabrication procedure follows the sequence of detection window creation, retaining frit formation, slurry packing, inlet and outlet frits fabrication and finally cutting of the retain frit. It is worth noting that the three parts (inlet frit, outlet frit and the detection window) of the capillary were not protected by polyimide coating making the capillary very fragile, so that its handling should be done very cautiously during or after packing. The fabrication of inlet and outlet frits was a key point for successful column preparation. Since the two frits were made by sintering the packed CSPs directly with a capillary burner, the appropriate burning time and temperature has to be determined to ensure the stability. Overheating would result in agglomeration of silica particles, resulting in blockage of the capillary while insufficient heating rendered the silica bed not robust enough for the CEC separation.

The CEC operation was performed on a Beckman MDQ CE system which is depicted by Figure 3-2.

Figure 3-2 CEC operation sketch

The mobile phase used was a mixture of organic modifier and buffers with the desired pH value. All the mobile phases were freshly prepared and degassed before use. Prior to the experiment, the packed capillary column was conditioned with the mobile phase for half an hour in order to support consistent electroosmotic flow (EOF). To avoid bubble formation (the most troublesome concern in CEC), both ends of the capillary were subjected to a pressure at 50 psi; moreover, a ramp time of the voltage should be set to let the pressure reach the desired value prior to the increase of voltage. In addition, high buffer concentration should be avoided to keep the current at low values to limit Joule heating effect.

3.3 EOF evaluation of the capillary columns

EOF is the primary driving force for neutral compounds in CEC and thus the

key factor in separation process. One of the most important properties of a packed CEC column is the ability to support stable and consistent EOF. There are many factors which influence the EOF stability such as the nature and surface area of the packed bed and packing material, the “frit effect” and Joule heating effect [52].

In this study, two background electrolytes (BGEs) (NaH_2PO_4 pH=7/ ACN and TEAA pH=7/ ACN) were employed to achieve the desired EOF. It was found that consistent cationic EOF could be obtained with both of these BGEs at low concentration (5 mM, pH 7) and the EOF with the phosphate BGE was much larger than that with the TEAA BGE (Figure 3-3). This can be explained by considering the effect of BGE on the charge of the silica and capillary surfaces. The adsorption of triethylammonium onto the silica surface reduces the surface anion charges and hence the reduced ζ potential which is the origin of EOF. The EOF of the packed capillary was found to be smaller than uncoated open tube capillary (Figure 3-3). It has been proven that the EOF in a packed capillary is generated mainly from the larger surface area of the solid packing surface compared with that of the capillary inner wall. Our result suggests that the tortuosity and the porosity of the flow channels between particles could reduce the EOF remarkably [187, 188]. The phosphate BGE was used for the rest of the studies in this work.

Figure 3-3 EOF for the **CSP1** packed capillary columns (a) (b) and open tube (c).
 Conditions: (a) 5 mM TEAA pH 7/ACN (50/50 v/v), 5 kV; (b) 5 mM NaH₂PO₄ pH 7/ACN (50/50 v/v), 5 kV; (c) 5 mM NaH₂PO₄ pH 7/ACN (50/50 v/v), 5 kV

The EOF of a “pure” packed-bed CEC should be directly proportional to the applied voltage if the CSPs bear good stability and the capillary columns have been well prepared. Herein, a good linear correlation ($Y = -0.04179 + 0.08469X$, Y is the EOF linear velocity, mm/s; X is the applied voltage, kV) was obtained by increasing the applied voltage from 2.5 kV to 15 kV for **CSP3** packed chiral capillary column (Figure 3-4). The result indicates that the capillary column was stable under the separation conditions used. A stable CEC column allows alteration of the applied voltage to optimize the separation resolution. This is simpler than altering the BGE (at least 1 h column condition is necessary by changing the BGE). High column efficiency (23000-25000 plates/m) was achieved for thiourea probe with 5 mM NaH₂PO₄ pH 7/ ACN (50/50 v/v) as the BGE.

Figure 3-4 EOF at different applied voltages. Conditions: 5 mM NaH_2PO_4 pH 7/ACN (50/50 v/v)

3.4 CEC enantioseparation with “click” chemistry derived perphenylcarbamoylated β -CD CSP3

The enantioseparation ability of the **CSP3** packed capillary was investigated by the separation of different racemic aryl alcohols (Figure 3-5).

Figure 3-5 Structures of racemic aryl alcohols

Since the same pressure was applied at both ends of the column, there was no net output driving force, and the result should only reflect the intrinsic effect of the EOF in the separation process. When voltage was applied, consistent cationic EOF was generated, showing that the triazolyl linkage would not affect the orientation of the EOF even though the linkage could be positively charged. After optimizing the separation conditions, most of the racemates could be resolved under different separation conditions. The enantioseparation results are shown in Table 3-1.

To ensure the reproducibility, each sample run was performed in triplicates. The mean separation factor (σ_α) and resolution (σ_{Rs}) were determined. It was found that the standard deviations of parameters were below 1.0×10^{-2} . Table 3-1 shows that high separation factor and good resolution were achieved using the current **CSP3** packed capillary under various operation conditions. **CSP3** afforded excellent enantioselectivity to the racemic aryl alcohols, which could be due to different interactive forces such as hydrophobic interaction, hydrogen bonding and π - π interaction between the analytes and the CSP in the reversed phase mode. The functionalization of the hydroxyl groups might also alter the hydrophobic chiral cavity which makes it suitable for some racemates. More discussion on the effect of analyte structure on the enantioseparation will be presented in later sections.

Table 3-1 Enantioseparation of aryl alcohols

Analytes	k'_1	k'_2	α	R_s
1 ^{a)}	0.65	1.08	1.66	1.86
2 ^{a)}	0.68	1.09	1.59	1.90
3 ^{a)}	0.71	0.78	1.10	0.79
4 ^{a)}	0.53	0.77	1.45	1.61
5 ^{a)}	0.28	0.36	1.25	0.95
6 ^{a)}	0.55	0.82	1.48	2.47
7 ^{a)}	0.63	1.09	1.73	2.67
8 ^{b)}	0.98	1.28	1.31	1.82
9 ^{a)}	1.22	2.42	1.98	4.61
10 ^{a)}	0.22	0.53	2.48	2.35
11 ^{b)}	0.83	0.96	1.15	0.96
12 ^{a)}	3.41	4.39	1.29	2.24

a) *Conditions*: NaH₂PO₄ buffer (5 mM, pH 7)/ACN(60/40 v/v); voltage, 10 kV

b) *Conditions*: NaH₂PO₄ buffer (5 mM, pH 7)/ACN(60/40 v/v); voltage, 5 kV

Note: Standard deviations of $\sigma_\alpha, \sigma_{R_s} < 1.0 \times 10^{-2}$

3.4.1 Effect of pH on enantioseparation

In order to investigate the influence of pH on the retention behavior of the current CSP, different pH values were varied from 5 to 8 in mobile phase consisting of 40% ACN and 60% 5 mM phosphates buffer. Since all the analytes are neutral alcohol compounds, sample 6 was chosen as the model analyte for subsequent experiments. The results obtained are shown in Figure 3-6.

Figure 3-6 Effect of pH on CEC enantioseparation of aryl alcohol sample 6. Conditions: mobile phase, 40% ACN and buffer (5 mM); voltage, 10 kV

As shown in Figure 3-6, the retention time of the analyte was marginally prolonged with the decrease of pH value. This is due to the enhanced EOF resulting from increased dissociation of acidic silanol (SiOH) moieties at the silica surfaces [189]. There was no significant difference in separation factors (1.45-1.49) and resolutions (2.35-2.48) since the analyte is neutral over the investigated pH range. Considering the analytical results and the neutrality of the analyte, we have used sample 6 buffered at pH = 7 to conduct further studies of the effects of other parameters.

3.4.2 Effect of buffer concentration on enantioseparation

Buffer concentration (ionic strength) plays an important part in CEC separation which could influence the EOF, separation factor, resolution and efficiency. A decrease in ionic strength will cause an increase in the double layer thickness,

which could result in an increase of EOF if there is no overlap of the double layer [190]. In this work, the effect of ionic strength was studied using six different NaH_2PO_4 concentrations (1.5, 2.5, 5, 7.5, 10 and 13 mM). The results are depicted in Figure 3-7. It is interesting to note that the elution time of the analyte did not show an obvious increasing tendency with the decrease of the buffer concentration. The retention time of the analyte slightly increased with the increment of ionic strength at a higher buffer concentration. However, at concentration below 5 mM, the retention time started to drop. Band-broadening of the peak was also observed, most notably at 1.5 mM. The resolution also decreased due to the band-broadening. This would suggest the strong overlap of the double layer in the silica pores which resulted in diminished EOF velocity and a degradation of the flow profile. This phenomenon was affirmed by the study of Cikalo *et al.* [190]. At 5 to 13 mM, retention time was prolonged with the increase of buffer concentration while the separation factors and resolutions were not significantly improved for this neutral alcohol. This might be due to the reduced EOF and Joule heating effect at higher ionic strength [191].

Figure 3-7 Effect of buffer concentration on the enantioseparation of sample 6. Conditions: mobile phase, 40% ACN and buffer (pH=7); voltage, 10 kV

3.4.3 Effect of applied voltage on enantioseparation

Two different voltages of 5 kV and 10 kV were applied to investigate the influence of voltage on the EOF and enantioseparation in a mobile phase containing 40% ACN and 60% 5 mM NaH_2PO_4 at pH = 7. Higher voltage significantly increased the velocity of EOF and reduced the retention time (Figure 3-8). EOF increased at higher voltage because the thickness of the electrical double layer and the positively charged counter-ions were kept constant at the same concentration of NaH_2PO_4 and pH value. Since the EOF was the only eluting force with neutral analytes, the retention time of the first eluted enantiomer almost doubled with the decrease of applied voltage from 10 to 5 kV. The separation factor and resolution also increased, especially the resolution (from 2.47 at 10 kV to 4.2 at 5 kV), which was due to the significantly longer analysis time, allowing sufficiently greater interaction between the analyte and CSP.

Figure 3-8 Effect of applied voltage on enantioseparation of sample 6. Conditions: mobile phase, 40% ACN and 5 mM NaH_2PO_4 (pH = 7)

3.4.4 Effect of organic modifier content on enantioseparation

The amount of organic modifier in the mobile phase strongly influences the elution time, separation factor and the resolution in CEC, and thus it is necessary to investigate the effect of this parameter on the enantioseparation. Acetonitrile was chosen as the organic modifier as it is one of the most widely used organic solvents in enantiomeric separation. The fraction of ACN in the mobile phase varied between 40 and 70%. Significant increase in the retention time and higher enantioseparation factor were found when the ACN concentration was decreased (See Figure 3-9). Resolution was also significantly improved with the decrease of ACN concentration. This was due to the increase of the hydrophobic interaction between the analyte and the CSP as a result of decreasing ACN concentration [192].

Figure 3-9 Effect of composition of organic modifier on enantioseparation of sample 6. Conditions: mobile phase, ACN and 5 mM NaH_2PO_4 (pH = 7); applied voltage, 5 kV

3.4.5 Effect of structural difference in the analytes

For cyclodextrin based CSPs, enantiomeric separations in the reversed phase mode are mainly achieved by the formation of enantioselective inclusion complexes. Hence, the analyte structure also plays an important role in enantioseparation process. In this work, the effects of the substituent groups near the chiral center and on the aromatic group were investigated (Figure 3-10). Sample 5, 6, 7, 9 and 12 were selected for comparison. From Figure 3-10, the retention time of samples in the above sequence (5, 6, 7, 9, 12) increased owing to accentuated hydrophobic interaction and increased interaction such as π - π effects between the analytes and CSP. The retention order of sample 5 to 7 indicated that the larger hydrophobic surface of the analyte, the higher the affinity, which was

also observed by Stalcup and coworkers [193]. Thermodynamic study by Shitangkoon *et al.* [194] also affirmed that the affinity was enhanced in the above sequence using heptakis(2,3-di-O-methyl-6-O-tert-butyltrimethylsilyl)-CD as the chiral stationary phase. This could explain that the separation factor and resolution increased from sample 5-7. Sample 9 depicted the highest separation factor, with the resolution two times higher compared to that of sample 7. This could be the result of the alkene moiety which afforded π - π and hydrophobic interactions. Sample 12 took on the longest retention time due to the additional π - π and hydrophobic interactions on account of the second aromatic ring and C=C double bond, the relatively low resolution may be attributed to the steric hinderance.

Figure 3-10 Effect of molecular structure on the enantioseparation. Conditions: mobile phase, 40% ACN and 5 mM NaH_2PO_4 (pH = 7); applied voltage, 10kV

Meanwhile, in order to check the applicability of our newly derived chiral phase for “real world” samples (e.g. pharmaceutical compounds), two drugs were tested on the **CSP3** packed capillary. Bendroflumethiazide is a drug used to treat mild

heart failure and hypertension. This weak acidic drug could be well resolved on **CSP3** packed capillary with 40% ACN and 60% 5 mM NaH₂PO₄ (pH = 7). A flavanone derivative, 7-methoxyflavanone, was also separated with a good resolution under the same separation condition (see Figure 3-11). This further reinforces the resolution ability of the **CSP3**.

Despite of the advantages of CEC, some drawbacks were found with this technique in our study. It requires much effort to optimize fabrication conditions for the packed capillary column, especially for the retaining frit creation step. Another drawback was found with the conservation of the packed capillaries. In this work, most of the packed capillaries cannot be reused after two weeks storage either with acetonitrile or with water.

Figure 3-11 Enantioseparation of pharmaceutical compounds on **CSP3**. Conditions: mobile phase, 40% ACN and 5 mM NaH₂PO₄ (pH = 7); applied voltage, bendroflumethiazide 10 kV; 7-methoxyflavanone 5 kV. Bendroflumethiazide: $\alpha = 1.21$, $R_s = 1.89$; 7-methoxyflavanone: $\alpha = 1.09$, $R_s = 1.51$

3.5 CEC Enantioseparation with “click” chemistry derived permethylated β -CD CSP2

Permethylated CD based CSPs have been widely used in many chromatographic methods such as HPLC, GC, OT-CEC for enantioseparation of various racemic compounds. In this section, the enantioseparation ability of a new permethylated **CSP2** packed capillary was investigated for the separation of some neutral flavonoids and racemic aryl alcohols (Figure 3-12) in 40% ACN/ 60% buffer (pH=7) at 5 kV or 10 kV.

3.5.1 Enantioseparation of flavonoids

Flavonoids, a class of plant secondary metabolites, are well-known for their anti-allergic, anti-inflammatory and anti-cancer activity [191]. Resolution of these analytes has been extensively investigated using cellulose and CD bonded CSPs in HPLC [192, 193]. In the present study, four different flavonoids were selected for enantioseparation (Table 3-2).

Figure 3-12 Structures of flavonoids and racemic aryl alcohols

Table 3-2 Enantioseparation results of different flavonoids on **CSP2**

Enantiomers	k_1	α	R_s	Voltage (kV)
Flavanone	1.81	1.03	0.83	5
	1.79	1.02	0.52	10
7-methoxyflavanone	2.15	1.15	1.63	5
	2.11	1.14	0.96	10
6-methoxyflavanone	2.35	1.08	1.11	5
	2.34	1.07	0.71	10
4'-hydroxyflavanone	1.86	1.06	0.64	5
	1.83	1.06	<0.4	10
7-hydroxyflavanone	1.82	1.05	0.66	5
	1.81	1.05	0.42	10

Conditions: mobile phase, 40% ACN and phosphate buffer (5 mM, pH=7)

It can be seen that the four flavonoids could be baseline or partially resolved on the **CSP2** with better resolution observed for the separation of the methoxyl

substituted flavonoids (1.63 for 7-methoxyflavanone and 1.11 for 6-methoxyflavanone) at 5 kV and decreased resolution observed for the hydroxyl substituted flavonoids (0.64 for 4'-hydroxyflavanone and 0.66 for 7-hydroxyflavanone). The retention factors for 7-methoxy and 6-methoxyflavanone were also higher than that of flavanone. However, substitution by hydroxyl groups reduced the retention time and the selectivity (like 4'-hydroxyflavanone and 7-hydroxyflavanone). These results suggested that lower polarity of the methoxyl group improves "host-guest" interactions with the mouth-extended permethylated CD, which could favor the well-known three-point interaction for enantioseparation. The difference of substitution positions and various substitution groups resulted in the different separation resolutions.

3.5.2 Enantioseparation of aryl alcohols, bupivacaine and DL-tyrosine

In addition to flavonoids, some other racemic compounds such as aryl alcohols, bupivacaine and DL-tyrosine were also tested on **CSP2** (Table 3-3).

In the CEC of neutral species, the solute and the mobile phase move in the same direction, and the sample components emerge in order of retention by the stationary phase. However, if the analyte is charged, the electrical migration will play an important role in the elution of the analyte besides EOF. Bupivacaine ($pK_a=8.1$) is a basic racemate mixture which is positively charged in the column pH, the direction of its electric migration was identical with the orientation of EOF. That is the reason why it presented the smallest retention factor ($k_I=0.43$).

DL-tyrosine is an amino acid (pI is around 6) negatively charged at $pH = 7$. Thus it was in the opposite orientation of the EOF and its electric migration resulted in the larger retention factor ($k_I=3.41$).

Table 3-3 Enantioseparation results of different enantiomers on **CSP2**

Enantiomers	k_I	α	R_s
1-phenylethanol ^(a)	0.74	1.09	0.87
1-phenyl-1-propanol ^(b)	1.11	1.10	1.12
1-(4-chlorophenyl)-ethanol ^(a)	1.49	1.07	0.96
4-(2-fluorophenyl)-1-buten-4-ol ^(a)	1.34	1.07	0.59
4-(4-methoxyphenyl)-1-buten-4-ol ^(a)	1.12	1.06	0.95
4-(4-phenylphenyl)-1-buten-4-ol	2.73 ^(a)	1.07 ^(a)	1.46 ^(a)
	2.71 ^(b)	1.09 ^(b)	0.89 ^(b)
DL-tyrosine ^(a)	3.41	1.05	0.68
Bupivacaine ^(a)	0.43	1.02	<0.3

Conditions: mobile phase, 40% ACN and phosphate buffer (5 mM, $pH=7$); applied voltage, (a) 5 kV (b) 10 kV

For neutral aryl alcohols which were only eluted by the EOF, due to their polarity and high solubility, the retention factors were below 1.5 with condition (a) (Table 3-3). The analyte 4-(4-phenylphenyl)-1-buten-4-ol was rendered with a larger retention factor due to the effect of the second aryl ring which increased hydrophobic interaction between the analyte and the **CSP2**. As EOF was the only eluting force, increasing applied voltage could lead to accentuated EOF hence the shorter elution time and lower resolution. This was confirmed by altering the applied voltage from 5 kV to 10 kV with the separation of 4-(4-phenylphenyl)-1-buten-4-ol. A higher resolution ($\alpha=1.46$) and larger retention factor ($R_s=2.73$) were obtained at 5 kV while these values decreased to 0.89 and

1.71 at 10 kV. Representative chromatograms of 1-phenyl-1-propanol and 7-methoxyflavanone on **CSP2** are shown in Figure 3-13.

Figure 3-13 Representative chromatograms on **CSP2**. Conditions: 5 mM NaH_2PO_4 pH 7/ACN (40/60 v/v), applied voltage 5 kV. 1-phenyl-1-propanol: $\alpha = 1.10$, $R_s = 1.12$; 7-methoxyflavanone: $\alpha = 1.15$, $R_s = 1.63$.

3.6 Enantioseparation with “click” chemistry derived native β -CD CSP1

The enantioseparation ability of **CSP1** was investigated by the separation of flavonoids and racemic aryl alcohols. However, the **CSP1** packed capillary did not show significant chiral recognition ability when aryl alcohols were used, presumably due to the lack of necessary structural features for a “tight” fit in the cavity of the more rigid, native CD. For flavonoids, **CSP1** presented quite different separation ability from the methylated CD CSP (**CSP2**) (Table 3-4).

The retention factors for all the flavonoids were much smaller than those on **CSP2**, reflecting the lower hydrophobicity of **CSP1** compared with the methylated CD CSP. The different chiral recognition behaviors of the two CSPs are particularly noteworthy. Although 7-methoxyflavanone and 6-methoxyflavanone

could be completely resolved on **CSP2**, no discrimination was observed on **CSP1**.

Table 3-4 Enantioseparation results of flavonoids on **CSP1**

Enantiomers	k_1	α	R_s	Voltage (kV)
Flavanone	0.85	1.41	4.2	5
	0.84	1.39	3.0	10
7-methoxyflavanone	0.68	<i>No separation</i>		5
	0.69			10
6-methoxyflavanone	0.61	<i>No separation</i>		5
	0.61			10
4'-hydroxyflavanone	0.90	1.60	5.4	5
	0.89	1.59	3.46	10
6-hydroxyflavanone	0.71	1.21	1.39	5
	0.71	1.20	0.85	10

Separation conditions: 5 mM NaH₂PO₄ pH 7/ACN (40/60 v/v)

For the flavanone and hydroxyl substituted flavanones, however, **CSP1** afforded much better separation even though the analytes were less retained on this CSP. This improved chiral recognition can be attributed to the stronger hydrogen bonding interactions. In addition, the better resolution of 4'-hydroxyflavanone relative to 6-hydroxyflavanone indicated that introducing a *p*-hydroxyl group could improve the chiral recognition for flavonoids on native CD CSPs. Representative chromatograms of flavanone and 6-hydroxyflavanone are shown in Figure 3-14.

Figure 3-14 Representative chromatograms on **CSP1**. Conditions: 5 mM NaH₂PO₄ pH 7/ACN (40/60 v/v), applied voltage 5 kV

3.6.1 Solvent effects

The separation of 4'-hydroxyflavanone at pH = 7 decreased with increasing acetonitrile concentration (40 to 60%) commensurate with the decreased in retention time (Figure 3-15). Surprisingly, the increase in the proportion of acetonitrile led to a slightly diminished EOF which usually prolongs retention. However, the influence of acetonitrile on the EOF was insignificant in comparison with that on the retention of analytes in this study.

Figure 3-15 Effects of ACN content on enantioseparation of 4'-hydroxyflavanone. Conditions: 5 mM NaH₂PO₄ pH 7/ACN, applied voltage 10 kV

The pH effect was insignificant on separation. On increasing the buffer pH from 5 to 8, the EOF increased slightly and there was little difference on the resolution. The pK_a of silanol groups is between 4 and 5, therefore, increasing the pH beyond 5 should not significantly enhance the surface charge.

3.7 Summary

Three chiral capillary columns were successfully fabricated with “click” chemistry derived **CSP1**, **CSP2** and **CSP3**. Consistent cationic EOF was obtained with phosphate and TEAA BGEs. The EOF with TEAA BGE was much smaller than phosphate BGE due to the reduced surface anions.

A good linear relationship between the EOF and applied voltage indicated good

reproducibility of the packed capillaries. High column efficiency of 23000-25000 plates/m was afforded for thiourea probe with 5 mM NaH₂PO₄ pH 7/ACN (50/50 v/v) as the BGE. “Pure” CEC enantioseparation of racemic aryl alcohols and some flavonoids was achieved by solely using EOF driving while keeping the pressure constant at both ends of the packed capillaries.

There was slight influence of buffer pH (5-8) on the EOF velocity and separation of neutral analytes. Decreasing applied voltage reduced the EOF and increased the resolution. Low buffer concentration (< 5 mM) resulted in peak band-broadening while high concentration brought on more Joule heating. The decrease of ACN content accentuated chiral resolution with only mildly reducing the EOF. The good separation results of the packed capillaries guaranteed that “click” chemistry was a facile and reliable approach for the preparation of cyclodextrin based chiral stationary phases. Detailed comparison of the three CSPs will be presented using the robust high performance liquid chromatography technique in the next chapter.

Although the packed-bed CEC affords high column efficiency and presents many advantages, there still exist many drawbacks in application of this technique such as difficulty in capillary fabrication and storage, and short shelf life of the packed capillaries.

Chapter 4*

Chiral HPLC with “click” chemistry derived cyclodextrin CSPs

* Much of this chapter has been published (*J. Chromatogr. A* 2009, 1216, 2388; *J. Chromatogr. A* 2010, 1217, 5103), permissions have been attached.

4.1 Introduction

A major concern in modern pharmaceutical industry is the chirality of the drug molecules due to their potentially different pharmacological activities in human bodies. The development of methods for enantiomeric purity check and pharmacokinetic investigations is attracting increasing interest [11, 12]. Although many separation techniques have appeared over the past decades such as SFC, GC, CE, MEKC and CEC, HPLC is still considered as the most commonly applied technique due to its versatility for a variety of analytes, flexibility for different separation conditions and simple operation requirements [48-52]. As it was discussed in Chapter 2, a new facile approach for the preparation of well-defined cyclodextrin CSPs has been developed via “click” chemistry with cuprous iodide-triphenylphosphine complex $[\text{CuI}(\text{PPh}_3)]$ as catalyst. The prepared **CSP1**, **CSP2** and **CSP3** afforded high stability and good enantioseparation ability when applied a series of racemic aryl alcohols and flavonoids in CEC (Chapter 3).

In this chapter, the enantioseparation abilities of **CSP1-3** were further investigated by separating a larger variety of racemic compound in the reversed phase mode HPLC.

4.2 Chiral HPLC with “click” chemistry derived native CD CSP1

Native CD bonded CSPs are invariably used for reversed phase chromatography due to the strong interaction between the hydrophobic CD cavity and non-polar organic solvents in normal phase mode, which would significantly reduce the

chiral resolution ability of CD. In this section, preparation of **CSP1** afforded a surface loading of $0.51 \mu\text{mol}\cdot\text{m}^{-2}$ (lower than that applied in CEC) and **CSP1** was packed into a stainless steel column with 4.6 μm i.d. and 15 cm length. The newly packed column was evaluated on a reversed phase mode for enantioseparation of twelve dansyl amino acids, five substituted phenyl and phenoxy modified propionic acids, five flavonoids, and some pharmaceutical compounds (Figure 4-1). In addition, some achiral compounds like xylenes and stilbenes were also separated using **CSP1**.

Figure 4-1 Structures of racemic compounds

4.2.1 Enantioseparation of dansyl amino acids

Dansyl amino acids are widely used as the analytes for the study of derived cyclodextrin selectors [196, 197]. In this study, **CSP1** was initially evaluated for the enantioseparation of twelve dansyl amino acids. The separation results and conditions are summarized in Table 4-1.

Table 4-1 Enantioseparation of dansyl amino acids on **CSP1**

Dns amino acids	k_1	k_2	α	R_s
Dns-Thr	1.75	2.48	1.41	2.32
Dns-Val	1.68	2.37	1.41	2.36
Dns-Met	1.99	2.83	1.42	2.39
Dns-Leu	3.68	7.35	1.99	5.08
Dns-Nle	2.34	3.49	1.49	3.21
Dns-Asp	2.39	2.93	1.23	0.95
Dns-Glu	2.21	3.71	1.22	1.25
Dns-Aba	1.31	1.62	1.24	1.21
Dns-Nva	1.23	1.60	1.30	1.42
Dns-Ser	1.53	1.92	1.25	1.36
Dns-Aca	2.85	4.90	1.72	3.5
Dns-Phe	2.50	3.76	1.50	2.43

Separation conditions: 1% TEAA buffer (pH=5.11)/methanol = 50:50 (v/v) flow rate = 0.7 mL/min

As shown in Table 4-1, all the dansyl amino acids are resolvable by **CSP1**. The resolutions of Dns-Thr, Dns-Val, Dns-Met, Dns-Leu, Dns-Nle, Dns-Aca and Dns-Phe were all above 2.0. The good separation results showed that the “click” chemistry derived native CD **CSP1** embodied good chiral recognition abilities towards dansyl amino acids. Good inclusion complexation occurred by the

penetration of the dimethylaminonaphthyl (Dns) group into the CD cavity, while its chiral center being close to the hydroxyl groups on the CD rims favored hydrogen bonds formation with the carboxylate and amine moieties [198]. The resolutions of Dns-Leu, Dns-NIe and Dns-Aca, 5.08, 3.21 and 3.5, respectively, were higher than those of other acids, indicating that the hydrophobicity of the substituted groups of the side chain on the chiral carbon played an important role in the enantioseparation process. The larger the hydrophobicity of this group, the better the chiral recognition. It was also interesting to note that the retention factors of Dns-Val and Dns-Leu (a branched alkyl group as side chain) were larger than those of Dns-NIe and Dns-Nva (a straight alkyl group as side chain). This result is suggestive that the influence of steric bulk of the side chain was preferable to the hydrophobicity effect for the retention based on the formation of inclusion complex, a finding which was consistent with Fujimura's study [199]. Compared with the ether linkages native β -CD CSP developed by Armstrong [200], the current **CSP1** afforded better chiral recognition ability to the dansyl amino acids. The presence of triazolyl linkage on the current CSP might also be favorable for the enantioseparation process. Herein, the nitrogen atoms in the linkage might provide additional hydrogen bonding interactions, which is the one of the most important interactions for the enantioseparation. Moreover, the relatively well-defined structure of the CSP could contribute to good enantioseparation as well.

4.2.1.1 Effect of pH on enantioseparation of Dns amino acids

As the dansyl amino acids are amphoteric analytes, their retention and separation resolution would be influenced by the buffer pH. To investigate the effect of pH on the retention and enantioselectivity of Dns amino acids, the buffer pH was increased from 4.11 to 7.11 with methanol and TEAA buffer (50:50, v/v) as the mobile phase and using Dns-Thr, Dns-Val, Dns-Met and Dns-Leu as model analytes. The results are summarized in Figure 4-2.

Figure 4-2 Effect of the buffer pH on the retention factor (top) and resolution (bottom). Separation conditions: 1% TEAA buffer/methanol = 50:50 (v/v); flow rate = 0.7 mL/min

Decreasing retention factors with increasing pH were observed for the four amino acids on **CSP1**. Accordingly, resolutions for all the model analytes also diminished with the increasing buffer pH in the current study. This phenomenon is consistent with other studies [201, 202]. Since the *pI* values of dansyl amino acids (*pK_a* of carboxylic group is about 3.0) are approximately 6.0, increasing pH may lead to greater extent of proton dissociation of the carboxyl groups. As a result, the increased hydration of the hydrophilic ionized moieties would be unfavorable for complex formation between the analytes and the CD hydrophobic cavity, leading to weakened retention. There is no significant loss of resolution, which is due to the sharper peaks obtained at higher pH.

4.2.1.2 Effect of organic modifiers on enantioseparation of Dns amino acids

Apart from the influence of buffer pH, the influence of organic modifier was also investigated in this study using the same four amino acids. The impact of varying methanol content on the retention and resolution are shown in Figure 4-3. The retention factors and the resolutions of the analytes declined with increasing methanol content. A widely acceptable view is that the hydrophobic interaction between the analyte and CD is affected in the presence of methanol [54]. The formation of the inclusion complex would diminish as the concentration of the organic modifier was increased.

Figure 4-3 Effect of MeOH content on the retention factor (top) and resolution (bottom). Separation conditions: 1% TEAA buffer (pH = 4.11)/methanol; flow rate = 0.7 mL/min

Besides methanol, acetonitrile (ACN) was also used in the current study. It was found that the influence of ACN on the retention factor was much stronger than methanol. ACN (20%) could not afford better separation results than 50% methanol (See Figure 4-4). This was probably attributed to the fact that ACN would afford higher probability of competition with the analytes in occupying the

CD hydrophobic cavity than methanol which is a protic solvent [49, 50].

Figure 4-4 Enantioseparation of Dns-Thr with different organic modifiers. Separation conditions: (i) 1% TEAA buffer/methanol = 50:50 (v/v) flow rate = 0.7 mL/min; (ii) 1% TEAA buffer/ACN = 60:40 (v/v) flow rate = 0.7 mL/min; (iii) 1% TEAA buffer/ACN = 80:20 (v/v) flow rate = 0.7 mL/min

4.2.2 Enantioseparation of hydroxyl, carboxylic acids and flavonoids

Apart from the dansyl amino acids, the resolving power of **CSP1** for a series of substituted phenyl and phenoxy group modified propionic acids and flavonoids was also examined using various separation conditions (Table 4-2).

Although native CD CSPs based on different linkages have been developed and applied for the enantioseparation of many racemic compounds, literature reports on

the enantioseparation of the propionic acids using native CD CSPs were limited. In this study, all five substituted phenyl and phenoxy group modified propionic acids could at least be partially resolved on this native CD bonded CSP using TEAA buffer and methanol as the mobile phase (Table 4-2). These results further confirmed the chiral recognition ability of the current CSP.

Table 4-2 Enantioseparation of racemic propionic acids and flavonoids

Compounds	k_1	k_2	α	R_s
Propionic acids				
2-POP A ^(a)	2.93	3.2	1.09	0.97
4-NPyP A ^(a)	6.09	6.54	1.07	0.90
3-CIPOP A ^(b)	5.26	5.65	1.07	0.67
4-OHPOP A ^(b)	3.13	3.35	1.07	0.67
2-OHPyP A ^(b)	4.85	5.15	1.06	0.51
Flavonoids				
Flavanone	1.08 ^(c) /6.19 ^(d)	1.71 ^(c) /6.90 ^(d)	1.58 ^(c) /1.11 ^(d)	2.26 ^(c) /1.31 ^(d)
4'-hydroxyflavanone	1.09 ^(c) /9.45 ^(d)	2.01 ^(c) /10.97 ^(d)	1.84 ^(c) /1.16 ^(d)	3.08 ^(c) /1.73 ^(d)
6-hydroxyflavanone	0.49 ^(c) /8.79 ^(d)	0.63 ^(c) /9.57 ^(d)	1.28 ^(c) /1.09 ^(d)	0.75 ^(c) /1.40 ^(d)
7-methoxyflavanone	0.73 ^(c) /5.77 ^(d)	0.73 ^(c) /6.08 ^(d)	1 ^(c) /1.05 ^(d)	0 ^(c) /0.65 ^(d)

Separation conditions: (a) 1% TEAA buffer (pH = 4.11)/methanol = 70/30 (v/v), flow rate = 0.3 mL/min; (b) 1% TEAA buffer (pH = 4.11)/methanol = 70/30 (v/v), flow rate = 0.4 mL/min; (c) water/ACN = 40/60, flow rate = 0.7 mL/min; (d) MeOH/water = 50:50 (v/v), flow rate 0.4 mL/min.

For the four flavonoids (shown in Table 4-2), flavanone, 4'-hydroxyflavanone and 6-hydroxyflavanone are separated using MeOH/ H₂O (50:50 v/v) as the mobile phase while 7-methoxyflavanone is partially resolved. The substituent group on the analyte molecular skeleton may play an important role in the separation process. Here, higher retention factor and resolution of 4'-hydroxyflavanone and

6-hydroxyflavanone could be attributable to hydrogen bonding between its hydroxyl group and the CSP, which would enhance separation. It is noteworthy that 4'-hydroxyflavanone could not be separated using the commercial Cyclobond I column from ASTEC (β -cyclodextrin, 250×4.0 mm i.d. $5 \mu\text{m}$ silica) as reported in the study by Krause and Galensa [203]. This would probably suggest that the triazolyl linkage of the **CSP1** could afford additional hydrogen bonding effect. For the case of 7-methoxyflavanone, the retention factor and resolution were lower than the other two flavonoids. Here, the presence of methoxyl group could accentuate steric hindrance for the phenyl group restricting interactions with the CD cavity. This phenomenon was also inferred in the report by Krause and Galensa [203]. In addition, the enantioseparations of flavanone and 4'-hydroxyflavanone appeared to be significantly improved on the **CSP1** column when the mobile phase comprising water and 40% ACN was applied (See Figure 4-5). However, 7-methoxyflavanone, which was partially resolved with the former mobile phase, could not be separated if 40% ACN and water was used as the eluent. Compared with the β -CD CSP developed by Liang *et al.* [133], the current **CSP1** provided much better chiral selectivity towards the flavanone and 6-hydroxyflavanone, the reason could be attributed to the high surface loading of CD selectors.

Figure 4-5 Enantioseparation of 4'-hydroxyflavanone with different conditions.
Separation conditions: (i) see (d) in Table 4-2; (ii) see (c) in Table 4-2

4.2.3 Enantioseparation of pharmaceutical compounds

In order to further evaluate the enantioselectivity of the novel **CSP1**, the enantioseparation of some pharmaceutical compounds like nimodipine (a dihydropyridine calcium channel blocker for treatment of high blood pressure), propranolol (a β -blocker for treatment of hypertension), brompheniramine (an antihistamine drug for treating hay fever) and bendroflumethiazide (a thiazide diuretic used to treat hypertension) were also carried out on **CSP1**. The separation results are summarized in Table 4-3.

Table 4-3 Enantioseparation of pharmaceutical compounds on **CSP1**

Pharmaceutical compounds	k_1	k_2	α	R_s
Nimodipine ^(a)	4.98	6.08	1.22	1.33
Propranolol ^(a)	6.45	6.90	1.07	0.82
Brompheniramine ^(b)	9.04	9.78	1.09	0.81
Bendroflumethiazide ^(a)	8.71	9.47	1.11	0.75
Chlorthalidone ^(c)	1.05	1.40	1.33	1.63
Cyclothiazide ^(d)	0.95	1.79	1.89	2.37
Tröger's base ^(e)	5.08	6.16	1.21	1.61

Separation conditions: (a) 1% TEAA buffer (pH = 4.11)/methanol = 70/30 (v/v), flow rate = 0.5 mL/min; (b) 1% TEAA buffer (pH = 4.11)/methanol = 60/40 (v/v), flow rate = 0.5 mL/min; (c) ACN/H₂O = 20/80 (v/v), flow rate = 0.7 mL/min; (d) ACN/H₂O = 40/60 (v/v), flow rate = 0.7 mL/min; (e) MeOH/H₂O = 50:50, flowrate = 0.5 mL/min

For nimodipine, the R_s value of 1.33 could be reached, which was higher than that by using a 25 cm long ether linkage native CD HPLC column; while for propranolol, the resolution on **CSP1** was lower than that on the 25 cm long ether linkage native CD column [93]. The separation power difference may be caused by the different structures of the two CSPs. The drugs brompheniramine, bendroflumethiazide and clopidogrel could also be partially resolved on this **CSP1** packed column. The results showed that the newly derived CSP embodied enantioselectivity to some “real world” pharmaceuticals in reversed phase mode HPLC. It is mentionable that enantioseparation of propranolol, bendroflumethiazide, chlorthalidone and Tröger's base were also performed in Liang's report using the similar CSP [133], however, the selectivities obtained in their study were lower than those on the current **CSP1**. The slightly better resolutions of bendroflumethiazide and chlorthalidone on their CSP were achieved

at the expense of increasing retention time, which could be also realized on the current **CSP1**.

Some typical chromatograms of the test analytes (Dns-Val, flavanone, 2-POP A and nimodipine) are shown in Figure 4-6. Moreover, the stability of the current CSP was ascertained by hundreds different sample injections under various separation conditions. It was found the standard derivation of the retention factors and resolutions were below 8×10^{-3} .

Figure 4-6 Typical chromatograms of the test analytes. Separation conditions: Dns-Val, 1% TEAA buffer/methanol = 50:50 (v/v) flow rate 0.7 mL/min; Flavanone, 1% TEAA buffer/ACN = 60:40 (v/v) flow rate 0.7 mL/min; 2-POP A, 1% TEAA buffer/methanol = 30:70 (v/v) flow rate 0.4 mL/min; Nimodipine, 1% TEAA buffer/methanol = 30:70 (v/v) flow rate 0.5 mL/min

4.2.4 Achiral separation of xylene and stilbene

Native CD CSPs could be employed to separate many structural and geometric isomers aside from enantiomers. Herein, as an extension of the previous work, achiral separation ability of **CSP1** was evaluated by resolving xylenes (o-, m- and p-xylene) and stilbene (cis- and trans-stilbene) in reversed mode HPLC (Figure 4-7).

Figure 4-7 Achiral separation of stilbene and xylenes on **CSP1**. Separation conditions: stilbene, MeOH/H₂O (v/v) = 60/40 flow rate = 0.7 mL/min; xylenes: MeOH/H₂O (v/v) = 50/50 flow rate = 0.7 mL/min

Longer retention was found with cis-stilbene than trans-stilbene, which might be the result of tighter inclusion due to the larger moiety of the two close phenyl rings. The good separation of xylenes indicates that inclusion complexation functions stereo selectively.

4.3 Chiral HPLC with permethylated CD CSP2 and perphenylcarbamoylated CD CSP3

As described in the introduction in Chapter 1, derivation of the -OH groups on the CD rings could accommodate various particular demands for separating specific chiral compounds and furnish distinct interactions with certain functional groups to produce highly effective separations for an array of different compounds. In this section, the permethylated and perphenylcarbamoylated CD CSP were applied in HPLC for enantioseparation of a wider range of racemic analytes. The scaled-up preparation of **CSP2** and **CSP3** afforded the surface loading of 0.42 and 0.10 $\mu\text{mol}\cdot\text{m}^{-2}$, respectively. Their enantio-recognition abilities were investigated using racemic aryl alcohols, flavonoids, β -blockers, α -ionone derivatives, atropine, derived thiazides, fenoterol, terbutaline, etilefrine, dobutamine and benzoin (See Figure 4-8) in reversed phase mode HPLC using methanol and acetonitrile as organic modifiers.

Figure 4-8 Structures of the chiral compounds

4.3.1 Enantioseparation with perphenylcarbamoylated CD CSP3

4.3.1.1 Enantioseparation of racemic aryl alcohols and flavonoids on CSP3

Most of the racemic aryl alcohols and flavonoids are neutral with the exception of naringenin and hesperetin which are only weakly acidic (See Table 4-4). Consequently, the separations could be carried out with water and the organic modifiers methanol (MeOH) or acetonitrile (ACN) as the eluting phase.

Table 4-4 Separation results for aryl alcohols and flavonoids on **CSP3**

<i>Compounds</i>	<i>k₁</i>	<i>k₂</i>	<i>α</i>	<i>R_s</i>	<i>Condition</i>
Aryl alcohols					
Aryl-OH 1	0.86	0.95	1.11	0.91	I
Aryl-OH 2	1.06	1.28	1.20	1.41	I
Aryl-OH 3	1.40	1.77	1.26	1.64	I
Aryl-OH 4	1.95	2.73	1.40	2.01	I
Aryl-OH 5	2.61	2.71	1.05	0.51	II
Aryl-OH 6	2.25 ^I , 3.39 ^{II}	2.93 ^I , 4.12 ^{II}	1.30 ^I , 1.22 ^{II}	3.56 ^I , 3.14 ^{II}	I and II
Aryl-OH 7	3.35	4.09	1.22	3.12	II
Aryl-OH 8	4.96	5.52	1.11	1.50	II
Flavonoids					
Flavanone	6.40	8.32	1.31	3.24	I
7-Methoxylflavanone	10.4	13.0	1.25	2.27	I
6-Methoxylflavanone	11.6	13.8	1.19	1.87	I
5-Methoxylflavanone	4.99	5.44	1.09	1.18	I
4'-hydroxylflavanone	5.70	7.80	1.37	1.98	I
6-hydroxylflavanone	2.27	2.54	1.12	1.37	I
Naringenin	11.6	12.9	1.11	0.54	I
Hesperetin	1.88	2.19	1.16	0.75	I

Conditions: I MeOH/H₂O = 50/50 (v/v), flow rate 0.7 mL/min; II ACN/H₂O = 20/80 (v/v), flow rate 0.7 mL/min

CSP3 displayed excellent resolution for all of the aryl alcohols with the exception of aryl-OH 1 and aryl-OH 5. The preferential inclusion sequence of organohalides by β -cyclodextrin was iodide > bromide > chloride > fluoride [204] and this was mirrored in the values of R_s and the retention factors for the series aryl-OH 1 - 4, indicating that inclusion was the predominant consideration for enantiomeric separation for these analytes. The better resolution of aryl-OH 6 compared with 1, suggested enhanced chiral discrimination by virtue π - π interactions between the allyl group of the guest and phenyl groups of the host. In addition, comparisons across the series aryl-OH 5 - 8 indicated that, perhaps surprisingly, substitution at either the para- or meta- positions did not have much influence on separation.

The unsubstituted flavanone registered the best resolution ($R_s = 3.24$). Good resolution was also obtained for the monosubstituted flavonoids. Monohydroxyl substitution on the analyte resulted in smaller retention factors relative to flavanone and the monomethoxyl substituted flavonoids and better resolution was achieved with 7-methoxyflavanone relative to 6-methoxyflavanone, with quite similar retention factors. The retention and resolution were sharply reduced for 5-methoxyflavanone. The same order was observed for the hydroxyl substituted flavonoids. The poor differentiation of naringenin and hesperetin was presumably the result of steric hindrance to inclusion for these bulkier analytes.

4.3.1.2 Enantioseparation of β -blockers, fenoterol, terbutaline, etilefrine and dobutamine

β -Blocker drugs are aromatic ethers bearing hydroxyl and isopropyl amino

functionality. Fenoterol, terbutaline, etilefrine and dobutamine could not be efficiently eluted with a simple MeOH/ H₂O or ACN/ H₂O mobile phase but separation was achieved using 1% TEAA buffer and either MeOH or ACN (Table 4-5).

Table 4-5 Separation of β -blockers, fenoterol, terbutaline, etilefrine and dobutamine

<i>Compounds</i>	<i>k₁</i>	<i>k₂</i>	<i>α</i>	<i>R_s</i>	<i>Condition</i>
Propranolol	8.19	10.13	1.24	1.36	I
Alprenolol	3.64	4.38	1.20	1.40	I
Pindolol	2.66	2.94	1.10	<0.4	I
Nadolol	1.76	2.38	1.35	1.99	I
Fenoterol	1.28	2.71	2.12	1.29	II
Terbutaline	0.04	0.08	2.00	0.62	II
Etilefrine	0.75	1.11	1.48	0.60	III
Dobutamine	1.11	1.31	1.18	0.96	III

Conditions: I MeOH/1%TEAA pH6.5 = 30/70 (v/v), flow rate 0.7 mL/min; II MeOH/1%TEAA pH 6.5 = 20/80 (v/v), flow rate 0.7 mL/min; III MeOH/1%TEAA pH6.5 = 50/50 (v/v), flow rate 0.7 mL/min

The retention factors for the four β -blockers decreased from propranolol to nadolol. The shorter retention of nadolol might be attributed to the presence of two hydroxyl groups which rendered it relatively hydrophilic and consequently less susceptible to inclusion. The same phenomenon was observed for the flavonoids. The interaction of these hydroxyl groups with the polar carbamoyl linkages on the CD rim might also contribute to the improved resolution of this analyte. Interestingly, nadolol was better resolved than propranolol despite the tighter binding of the latter's naphthyl rings in the β -CD cavity. We have observed this previously with urea bonded CSPs [119].

The structurally similar drugs fenoterol, terbutaline, etilefrine and dobutamine could also be resolved by **CSP3** (Table 4-5). Fenoterol and dobutamine registered longer retention factors with better resolutions than did terbutaline and etilefrine under the same conditions presumably due to the lack of a second phenyl ring in the latter analytes.

4.3.1.3 Enantioseparation of other aromatic pharmaceutical compounds

A number of other aromatic pharmaceutical compounds were also resolved on the **CSP3** (Table 4-6).

Table 4-6 Separation results for other pharmaceuticals on **CSP3**

<i>Chiral drugs</i>	<i>k₁</i>	<i>k₂</i>	<i>α</i>	<i>R_s</i>	<i>Condition</i>
Atropine	0.74	1.21	1.63	2.89	III
<i>p</i> -Chlorophenyl-pyrimidine	1.11	1.32	1.18	0.67	III
Bendroflumethiazide	1.86	2.38	1.28	2.82	IV
Cyclothiazide	k ₁ = 1.73 k ₂ = 1.94 k ₃ = 2.08 k ₄ = 3.32				IV
Benzoin	12.45	12.96	1.04	0.41	V

Conditions: III MeOH/1%TEAA pH 6.5 = 50/50 (v/v), flow rate 0.7 mL/min; IV ACN/H₂O = 40/60 (v/v), flow rate = 0.7 mL/min; V MeOH/H₂O = 20/80 (v/v) flow rate = 0.7 mL/min

Atropine is a tropane alkaloid used as a cycloplegic. It was fast eluted and well resolved on the **CSP3** using TEAA buffer and methanol. This good separation could be attributable to the inclusion of the phenyl ring and concomitant hydrogen bonding of the rim carbamoyl groups to the tertiary amine. The phenylcarbamoylated CSP also afforded good chiral discrimination to bendroflumethiazide and cyclothiazide in which the chiral carbon is located adjacent to hydrogen bonding functionality. Benzoin was the longest retained of

these compounds, presumably because of the two phenyl rings, and with the poorest discrimination, highlighting that long retention may not lead to better resolution.

Some representative chromatograms on **CSP3** are depicted in Figure 4-9.

Figure 4-9 Typical chromatograms on current **CSP3**. Separation conditions: (a) MeOH/TEAA (pH 6.5) = 30/70 (v/v), flow rate 0.7 mL/min; (b) MeOH/H₂O = 50/50 (v/v), flow rate 0.7 mL/min; (c) ACN/ H₂O = 30/70 (v/v), flow rate 0.8 mL/min; (d) MeOH/TEAA (pH 5.2) = 50/50 (v/v), flow rate 0.7 mL/min

4.3.1.4 Influence of organic modifiers on enantioseparation

The choice of an appropriate organic modifier for certain analytes is important for separation method development and optimization. The most commonly used organic modifiers are MeOH and ACN. In this study, the influence of organic

modifiers on enantioseparation was carried out by altering the content of ACN or MeOH in the mobile phase. As expected, all the analytes were eluted more rapidly when the organic modifier content increases and the resolution decreased correspondingly. ACN and MeOH have different effects on the elution rates and chiral discrimination. In the current work, we found that ACN generally resulted in faster elution while MeOH usually afforded better chiral discrimination. This was presumably the result of stronger competition by ACN relative to MeOH for inclusion in the hydrophobic CD cavity. For example, excellent separation of flavanone was achieved using MeOH and H₂O as the mobile phase, while ACN and H₂O only partially resolved this analyte even at a longer analysis time (Figure 4-10 (a)). However, the reversed was observed with bendroflumethiazide (Figure 4-10 (b)) possibly due to the highly polar nature of this analyte and its strong hydrogen bonding with MeOH/ H₂O.

Figure 4-10 Influence of organic modifiers on the enantioseparation. (a) flavanone; (b) bendroflumethiazide

Although **CSP3** could resolve a broad range of racemic analytes, the resolutions were lower than those achievable on the urea bonded perphenylcarbamoylated CSPs reported by our groups previously [122, 123]. On one hand, the surface loading of **CSP3** was lower than the urea bonded CSP due to the steric hindrance of bulky phenylcarbamoyl groups during the reaction; on the other hand, the carbonyl and amino moieties on the urea linkages supplies additional dipole-dipole and hydrogen bonding effects. Furthermore, the residual alkynyl groups on the

newly derived CSPs may afford adverse effects on the separation efficiency.

4.3.2 Enantioseparation with permethylated CD CSP2

The permethylated CD **CSP3** only exhibited chiral recognition towards some of the flavonoids and the two α -ionone derivatives in reversed phase mode HPLC (Table 4-7).

This lack of discrimination for **CSP2** relative to the perphenylcarbamoylated **CSP3** presumably reflected the decrease in H-bonding functionality on the CD rim and absence of potential π - π interactions. Of the five flavonoids, only 7-methoxyflavanone could be baseline resolved. Separation decreased on changing the substituent from methoxyl to hydroxyl while the retention factors remained comparable (6-, 7-methoxy and 4', 6-hydroxyflavanones), again illustrating that tight inclusion (reflected in a large retention factor) does not always result in improved resolution.

Table 4-7 Enantioseparation results on **CSP2**

compounds	k_1	k_2	α	R_s	Condition
7-methoxyflavanone	10.31	10.8	1.06	1.52	VI
6-methoxyflavanone	10.8	11.7	1.09	0.87	VI
Flavanone	7.9	8.27	1.05	0.75	VI
5-methoxyflavanone	6.07	6.27	1.03	<0.4	VI
6-hydroxyflavanone	11.7	12.1	1.05	0.45	VI
4'-hydroxyflavanone	11.2	11.8	1.04	<0.4	VI
α -ionone	2.34	2.88	1.23	2.48	VII
3-methyl- α -ionone	2.88	3.85	1.34	3.26	VII

Separation conditions: VI MeOH/H₂O = 30/70 (v/v), flow rate = 0.5 mL/min; VII MeOH/H₂O = 40/60 (v/v) flowrate = 0.5 mL/min

CSP2 could resolve α -ionone and 3-methyl- α -ionone which bear no aromatic groups. These compounds were not resolved on phenylcarbamoylated **CSP3**, presumably due to the absence of π - π interactions. It was not obvious why these alicyclic analytes could be resolved using permethylated CD column and not by a perphenylcarbamoylated CD column, but it suggested that a relatively strong, non-specific hydrophobic interaction between these analytes and the rim methoxyl groups. Representative chromatograms of 3-methyl- α -ionone and 7-methoxyflavanone are shown in Figure 4-11.

Figure 4-11 Separation of 3-methyl- α -ionone and 7-methoxyflavanone on **CSP2**. Separation conditions: (a) MeOH/H₂O = 40/60 (v/v), flow rate 0.5 mL/min; (b) MeOH/H₂O = 30/70 (v/v), flow rate 0.8 mL/min

4.4 Comparison of CSP1, CSP2 and CSP3

As mentioned in previous sections, different substitutions afforded various chiral recognition abilities attributed to the diverse interactions between CDs and analytes. Generally, hydrogen bonding was the most important interaction in enantioseparation with CD selectors apart from the inclusion complexation. That's the reason why the native CD **CSP1** and perphenylcarbamoylated CD **CSP3** afforded more powerful enantio-recognition ability over the permethylated CD **CSP2**.

4.4.1 Selectivity of amphoteric, acidic compounds and ionones

The different selectivities of the three CSPs for Dns-Aba, Dns-Nva, Dns-Ser, Dns-Aca, Dns-Phe, 2-POP A, 3-CIPOP A, 2-OHPyP A, α -ionone and 3-methoxyl- α -ionone are shown in Figure 4-12.

Figure 4-12 Selectivities of anionic and ampholytic acids as well as ionones

In reversed phase mode HPLC, native CD **CSP1** exhibited resolution ability towards various aromatic acids, particularly dansyl amino acids. These analytes were not resolved using either **CSP2** or **CSP3**. It was evident that separation of aromatic carboxylic acids depended on both inclusion complex formation between CD cavity and aromatic rings and H-bonding originating from the hydroxyl groups of the CD rings. The methoxyl groups on **CSP2** only provided hydrophobic and steric interaction sites. Although **CSP3** could offer H-bonding sites, the interaction

arises from the carbamido -NH which is distinct from the -OH in **CSP1**. With regard to the two fragrance molecules α -ionone and 3-methyl- α -ionone, only the permethylated CD **CSP2** could resolve them with good selectivity. As the carbonyl groups on the two ionones is far from the respective chiral centers, hydrogen bonding sites on **CSP1** and **CSP3** would not be able to participate in the chiral recognition process while hydrophobic and steric effects from the methylated CD on **CSP2** would still function well in this situation.

4.4.2 Selectivity of flavonoids and racemic aryl alcohols

Even if the three CSPs all afforded chiral selectivity towards flavonoids, the actual values varied with different CSPs. As shown in Figure 4-13, **CSP2** and **CSP3** could resolve a broader range of flavonoids than **CSP1** with better selectivity on **CSP3**. However, **CSP1** affords extraordinary selectivity for flavanone, 4'-hydroxyflavanone and 6-hydroxyflavanone, in comparison with the other two CSPs, depicting the highest selectivity for 4'-hydroxyflavanone. Methoxyl substitution on the flavanone skeleton reduces the selectivity of **CSP1**. Neither **CSP1** nor **CSP2** were able to resolve the bulky substituted flavonoid naringenin. In addition, **CSP3** demonstrated good chiral selectivity towards many racemic aryl alcohols in contrast to **CSP1** and **CSP2** where no chiral discrimination was found. The broader recognition ability of **CSP3** could be due to the presence of the multiple interactions including π - π , H-bonding and dipole-dipole effects ascribable to the phenylcarbamoyl groups.

Figure 4-13 Selectivities of flavonoids and racemic aryl alcohols

4.4.3 Selectivity of other pharmaceutical compounds

In addition to the chiral compounds mentioned above, some other commonly used pharmaceutical compounds such as nimodipine, chlorthalidone, Tröger's base, propranolol, pindolol, terbutaline, atropine and bendroflumethiazide were selected for the comparison (Figure 4-14).

Figure 4-14 Selectivities of pharmaceutical compounds

As shown in Figure 4-14, of the nine selected pharmaceutical drugs, six could be resolved by **CSP1** and five were separated by **CSP3**, and only one was discriminated by **CSP2**. Nimodipine, chlorthalidone and Tröger's base were separated on **CSP1** exclusively; pindolol, terbutaline and atropine were only resolved on **CSP3**; while propranolol and bendroflumethiazide could be recognized on **CSP1** and **CSP3**. Separation of nimodipine analogs such as felodipine, nitrendipine and nifedipine can be effected on **CSP1** as well. However, it was found that **CSP1** could only resolve nimodipine even if the analogs have very similar structures. The results revealed that there were no universal chiral selectors; discrimination of different analytes required different chiral selectors and optimization of eluting phases.

4.5 Comparison of CEC and HPLC

In Chapter 3, we presented the CEC application of the “click” chemistry derived CSPs. Although the capillaries possess different inner diameter and effective length from the HPLC column and the CSPs had unequal surface loadings, the comparison of CEC and HPLC may still provide some useful insights.

CEC technique required very small amount of organic solvent and buffers. The column efficiency with thiourea probe ($\text{NH}_2=\text{CS}=\text{NH}_2$, 23000-25000 plates/m) was much higher than HPLC (2060-2100 plates/meter) on the account of the favorable plug flow profile of the electroosmotic flow (EOF). The high efficiency rendered CEC with better chiral resolution ability over the HPLC technique despite of the capillary's shorter column bed. Figure 4-15 compares the resolutions attained for selected analytes on CEC and HPLC.

Figure 4-15 Comparison of the resolutions between CEC and HPLC

For the two racemic aryl alcohols on **CSP3**, CEC with ACN and phosphate buffer afforded much higher resolutions over HPLC with ACN and water. Since these analytes are neutral, phosphate buffer did not exert significant influence on the resolution. The higher resolution was attributed to the high efficiency of CEC. The same trend was found with **CSP1** and **CSP2** for the separation of flavonoids. Moreover, **CSP2** could partially separate several racemic aryl alcohols in CEC while no separation was found with HPLC.

Although packed-bed CEC was superior to HPLC in separation efficiency and resolution, some drawbacks still restrict its applications. CEC capillaries with greater stability and longer shelf life are required.

4.6 Summary

Three CSPs derived from “click” chemistry were successfully applied in reversed phase mode HPLC for enantioseparation of a variety of racemic compounds including dansyl amino acids, hydroxyl and carboxylic acids, flavonoids, aryl alcohols, β -blockers, fragrance ionones and other pharmaceutical chiral compounds such as brompheniramine, nimodipine, atropine, benzoin, chlorthalidone, bendroflumethiazide, cyclothiazide, fenoterol, tebutaline, dobutamine, etilefrin and Tröger’s base.

Different functional groups on the CD rims afforded diverse enantiorecognition abilities. Thus, native CD resolved dansyl amino acids, hydroxyl and carboxylic acids very well; permethylated CD could separate ionones while

perphenylcarbamoylated CD showed the most powerful enantiorecognition ability to racemic aryl alcohols.

Organic modifiers played a crucial role in enantioseparation. Acetonitrile was a stronger eluting solvent than methanol due to its stronger affinity for the CD hydrophobic cavity while methanol being a protic solvent acted very differently from acetonitrile. For the native CD **CSP1**, acetonitrile was better than methanol whereas the reversed situation was favorable for **CSP2** and **CSP3** for most of the studied analytes.

The good stability and separation results achieved on the prepared CSPs affirmed the reliability of “click” chemistry on the preparation of cyclodextrin chiral stationary phases.

Chapter 5*

Efficient enantioseparation on “click” hindered cyclodextrin chiral stationary phases (CSP4 and CSP5) by HPLC

* Much of this chapter has been published (*J. Chromatogr. A* 2010, 1217, 7878), permission has been attached.

5.1 Introduction

Resolution of racemic molecules by the formation of transient, diastereomeric cyclodextrin-analyte complexes is well known [90, 93, 96]. In Chapter 4 we have detailed the HPLC enantioseparations on the “click” chemistry derived native cyclodextrin chiral stationary phase (**CSP1**) tethered to silica via a single triazolyl linkage. This CSP exhibited reasonable enantiorecognition of dansyl amino acids, flavanone and monohydroxyl substituted flavonoids in reversed phase mode. However, the synthesis of the intermediate mono-6-tosyl- β -CD (a prerequisite for the the syntheses of monoazido-CD derivatives) gave relatively low yields. Moreover, the single triazolyl linkage might become unstable after long operations with buffers.

To afford a more efficient preparation procedure and improve the stability of the CSP in acidic and basic media, we prepared and investigated two CSPs (Figure 5-1) bonded to silica with multiple triazolyl linkages in HPLC. The detailed preparation procedures and characterization data have been elaborated in Chapter 2 and Chapter 6. **CSP4** was prepared by immobilizing heptakis-N₃-CD by multiple “click” chemistry reactions with alkyne functionalized silica. **CSP5** was prepared using the same Cu (I) catalyzed reaction but of heptakis-N₃-Ph-CD and so does not bear any free hydroxyl groups. In both cases the smaller CD rim was almost closed by the multiple triazolyl linkages. These materials demonstrated quite different enantioseparation properties from each other and from the previously described “click” derived CD CSP.

The two stable stationary phases were sterically congested at the smaller rim of the CD and exhibited good enantirecognition in high performance liquid chromatography. Over forty racemic compounds (Figure 5-2) including hydroxyl, carboxylic and dansyl amino acids, aryl alcohols, flavonoids, β -blockers and β -agonists, thiazides, indoprofen, ketoprofen, Tröger's base and atropine were resolved or partially separated on the two CSPs in reversed phase mode. The free hydroxyl CSP afforded enantioselectivities to indoprofen, ketoprofen, Tröger's base, hydroxyl, carboxylic and dansyl amino acids, better than did the phenylcarbamoylated CSP while the latter was better at separating aryl alcohols, flavonoids, β -blockers and β -agonists.

Figure 5-1 Structures of **CSP4** and **CSP5**

Figure 5-2 Structures of the studied racemic compounds

5.2 Enantioseparations on CSP4 and CSP5

The enantiorecognition abilities of the two newly prepared CSPs were investigated by HPLC enantioseparation of various racemic compounds in reversed phase mode. **CSP4** could afford enantioselectivity towards twenty four of the analytes investigated while twenty seven were partially or baseline separated on **CSP5** under different separation conditions, this added up to coverage of all the studied racemic pairs.

5.2.1 Chiral HPLC with CSP4

CSP4 bears free hydroxyl groups only on the larger rim of the CD. It afforded chiral recognition ability to flavonoids, hydroxyl, carboxylic and dansyl amino acids, indoprofen, ketoprofen, brompheniramine, cyclothiazide and Tröger's base. The retention and resolution parameters are listed in Table 5-1.

Table 5-1 Enantioseparation results on **CSP4**

<i>Analytes</i>	<i>k₁</i>	<i>k₂</i>	<i>α</i>	<i>R_s</i>	<i>conditions</i>
Flavanone	22.4	23.1	1.03	0.41	(a)
6-methoxyflavanone	19.2	20.8	1.08	1.09	(a)
7-methoxyflavanone	18.7	20.4	1.09	1.15	(a)
5'-methoxyflavanone	15.3	17.5	1.14	1.65	(a)
4'-hydroxyflavanone	24.1	25.4	1.05	0.64	(a)
6-hydroxyflavanone	23.3	24.5	1.05	0.62	(a)
Dns-Thr	2.92	3.46	1.18	0.91	(b)

Dns-Val	2.67	3.29	1.23	1.45	(b)
Dns-Leu	3.05	3.33	1.09	0.61	(b)
Dns-Glu	3.81	4.51	1.19	1.35	(b)
Dns-Nva	2.39	2.68	1.11	0.51	(b)
Dns-Ser	2.46	2.81	1.14	0.53	(b)
Dns-Phe	13.8	14.5	1.05	0.40	(b)
2-POP A	2.02	2.44	1.21	2.00	(c)
2-OHPP A	2.42	2.67	1.10	0.98	(c)
2-PB A	3.21	3.62	1.13	1.33	(c)
3CIPOP A	3.52	3.82	1.09	1.06	(c)
4-NPOP A	5.50	5.83	1.06	1.17	(c)
4-OHPOP A	1.37	1.58	1.15	1.25	(c)
Brompheniramine	2.38	2.56	1.07	0.42	(d)
Indoprofen	8.46	9.06	1.07	0.73	(d)
Ketoprofen	8.37	8.87	1.06	0.65	(d)
Cyclothiazide	2.12	2.78	1.31	1.05	(b)
Tröger's base	16.6	19.7	1.18	1.64	(a)

Separation conditions: flow rate = 0.7 mL/min (a) MeOH/H₂O (v/v) = 35/65; (b) ACN/TEAA (pH = 5.2) (v/v) = 35/65; (c) MeOH/TEAA (pH = 5.2) (v/v) = 50/50; (d) ACN/TEAA (pH = 5.2) (v/v) = 25/75

Six flavonoids including those bearing -OCH₃ and -OH groups on the bicyclic rings or -OH on the single phenyl ring were all resolved using MeOH and water as the eluting phase. However, the chiral selectivities were modest and only

5'-methoxyflavanone could be baseline separated. The three flavonoids with -OCH₃ moieties linked to the bicyclic rings were eluted faster but better resolved on **CSP4** whilst the unsubstituted flavanone had the lowest resolution. This profile was different to the results previously obtained with **CSP1**. **CSP1** demonstrated good chiral selectivity towards flavanone and hydroxyl substituted flavonoids under the same conditions while **CSP4** appeared to be better at resolving methoxyl functionalized flavonoids. The latter CSP had a sterically restricted CD rim which appeared to be important to the “host-guest” inclusion of methoxyl substituted flavonoids. Since it was believed almost impossible for flavonoids to penetrate the CD cavity via the primary CD face using **CSP4**, the inclusion phenomenon was supposed to take place at the larger CD rim which resulted in a different inclusion mode compared to **CSP1**. Meanwhile, the much lower resolution of flavanone and hydroxyl substituted flavonoids on **CSP4** revealed the significance of the primary -OH in the chiral discrimination process.

Seven dansyl amino acids were partially resolved on **CSP4** with Dns-Phe exhibiting the longest retention time. In reversed phase mode, retention of analytes is primarily controlled by inclusion complex formation while the predominant determinants for chiral recognition are hydrogen bonding and steric interactions. Dns-Phe has two aromatic moieties which could significantly increase its retention, but resolution was modest. Compared with **CSP1**, the current **CSP4** afforded much lower enantioselectivity to all the studied dansyl amino acids (Figure 5-3). The poorer enantiomeric discrimination of dansyl amino acids with **CSP4** may be due

to the complex structures of the CSP.

Figure 5-3 Comparison of the selectivities of Dns amino acids and substituted phenyl acids on **CSP4** and **CSP1**

Five pharmaceutical compounds, brompheniramine, indoprofen, ketoprofen, cyclothiazide as well as Tröger's base, could also be at least partially resolved on **CSP4**. Indoprofen and ketoprofen were not separated on **CSP1** with the single triazolyl linkage, but could be partially resolved on **CSP4**. In general, however, resolution was not as good with **CSP4** than with **CSP1**. The former could not resolve the chiral drugs nimodipine, propranolol, bendroflumethiazide and chlorthalidone. Although Tröger's base was baseline resolved on this CSP, as it was on **CSP1**, the retention time was significantly longer. These results indicated the important interactions of these chiral analytes with the CD primary -OH.

Compared with the CSP by Liang *et al.* [133], the selectivities and resolutions of flavanone, 6-hydroxyflavanone and brompheniramine on **CSP4** were lower while

the separations of 6-methoxyflavanone and Tröger's base were better than the CSP developed by Liang *et al.* with the same retention factors. Hence, choice of an appropriate CSP for individual analytes is important even if the CSPs contain same chiral selectors.

Typical chromatograms of 2-PB A, Dns-Glu, indoprofen, 2-POP A, Tröger's base and 5-methoxyflavanone on **CSP4** are depicted in Figure 5-4.

Figure 5-4 Representative chromatograms on CSP4. Separation conditions and results see Table 1

5.2.2 Chiral HPLC with CSP5

Phenylcarbamoyl groups provide π - π and dipole-dipole interactions which are not available on unmodified CD CSPs. Twenty seven racemic compounds including flavonoids, aryl alcohols, β -blockers and β -agonists, atropine, *p*-chlorophenyl-pyrimidine, cyclothiazide and bendroflumethiazide were resolved or partially separated on **CSP5** in reversed phase mode HPLC (Table 5-2).

Table 5-2 Enantioeseparation results on CSP5

<i>Analytes</i>	<i>k₁</i>	<i>k₂</i>	<i>α</i>	<i>R_s</i>	<i>conditions</i>
Flavanone	4.79	5.53	1.15	2.54	(a)
6-methoxyflavanone	7.43	8.37	1.13	2.12	(a)
7-methoxyflavanone	7.55	9.27	1.22	2.91	(a)
5-methoxyflavanone	4.03	4.10	1.02	1.06	(a)
4'-hydroxyflavanone	3.61	3.98	1.10	1.51	(a)
6-hydroxyflavanone	3.81	4.07	1.07	1.34	(a)
Aryl-OH 1	1.94	2.18	1.12	1.84	(b)
Aryl-OH 2	2.76	2.93	1.06	1.24	(b)
Aryl-OH 3	1.93	2.18	1.13	1.96	(b)
Aryl-OH 4	3.19	3.64	1.14	2.41	(b)
Aryl-OH 5	1.81	2.05	1.13	1.97	(b)
Aryl-OH 6	0.99	1.03	1.04	0.42	(b)
Alprenolol	1.66	2.02	1.20	2.02	(c)

Metoprolol	0.95	0.99	1.04	0.70	(c)
Nadolol	$k_1=0.41$	$k_2=0.58$		$k_3=0.99$	(c)
Pindolol	1.04	1.12	1.07	0.88	(c)
Labetalol	3.02	3.23	1.07	<0.3	(c)
Propranolol	3.24	4.10	1.27	2.20	(c)
Acebutolol	1.29	1.33	1.03	0.71	(c)
Fenoterol	1.24	2.92	2.35	2.70	(c)
Terbutaline	0.61	1.13	1.84	2.26	(d)
Dobutamine	1.09	1.80	1.84	1.86	(e)
Eltilerfrine	3.03	3.28	1.08	0.77	(f)
Atropine	0.81	1.44	1.78	3.57	(f)
Brompheniramine	3.10	3.21	1.03	<0.3	(f)
<i>p</i> -Chlorophenylpyrimidine	2.12	2.40	1.13	0.96	(f)
Cyclothiazide	5.31	5.83	1.10	1.08	(g)
Bendroflumethiazide	5.80	6.31	1.09	1.48	(g)

Separation conditions: flow rate = 0.7 mL/min (a) MeOH/H₂O (v/v) = 50/50; (b) ACN/H₂O (v/v) = 35/65; (c) MeOH/TEAA (pH = 4.2) (v/v) = 35/65; (d) MeOH/TEAA (pH = 4.2) (v/v) = 5/95; (e) MeOH/TEAA (pH = 4.2) (v/v) = 50/50; (f) ACN/TEAA (pH=4.2) (v/v)=35/65; (g) ACN/H₂O (v/v)=40/60

CSP5 afforded better separation for most flavonoids compared to **CSP4**, presumably because of π – π interactions between the CD phenylcarbamoyl groups and the aromatic analytes. 6-Methoxyflavanone and 7-methoxyflavanone were more strongly retained presumably because of the lipophilicity of the methoxyl

groups. However, long retention does not always correlate with better resolution, as was observed for flavanone and 6-methoxyflavanone. The latter had a shorter retention time but was better resolved. Of the three methoxyl substituted flavonoids, 5-methoxyflavanone had the shortest retention time and poorest resolution.

Enantioseparation of some β -blockers and β -agonists were achieved using TEAA buffer. Alprenolol and propranolol showed enhanced enantioseparation relative to similar compounds as expected. The naphthyl group in propranolol fitted tightly into the β -CD cavity. It appeared that the ortho ethylene benzene group of alprenolol adopted a planar (naphthalene-like) conformation and was similarly tightly bound. Fenoterol and terbutaline exhibited good resolution, perhaps because the chiral center was located at the α -position thereby facilitating the three point interaction mode. There should be four peaks for nadolol due to the two chiral carbons located in its structure, however, only three peaks obtained in this study indicating **CSP4** could only afford enantioselectivity to one chiral carbon.

The good resolving ability of **CSP5** was further investigated with atropine, brompheniramine, *p*-chlorophenylpyrimidine, cyclothiazide and bendroflumethiazide. Atropine was baseline resolved with a high resolution of 3.57, presumably due, in part, to the location of the chiral center on the α -position of the aromatic ring responsible for inclusion, and the various functional groups in the structure supplying H-bonding and dipole-dipole interactions. The chiral centers of

p-chlorophenylpyrimidine and brompheniramine are also located at the α -position, but both of these analytes lack the additional dipole-dipole interaction functionality and were not as well resolved. Cyclothiazide and bendroflumethiazide bear strongly H-bonding moieties with -NH and SO₂NH- groups adjacent to the chiral carbon and were relatively well resolved.

Representative chromatograms of 7-methoxyflavanone, Aryl-OH, propranolol, fenoterol, atropine and brompheniramine are depicted in Figure 5-5.

Figure 5-5 Representative chromatograms on **CSP5**. Separation conditions and results see Table 2

5.2.3 Solvent effects

The eluting phase comprises of organic modifiers (ACN and MeOH) and water or TEAA buffer. Some neutral and weak acidic or basic racemic compounds such as alcohols, flavonoids, thiazides and Tröger's base were eluted simply with ACN/ MeOH and water. However, TEAA buffer was required for elution of ionic racemates such as hydroxyl, carboxylic and dansyl amino acids, β -blockers, β -agonists, and atropine.

In general, increasing the proportion of organic modifier reduced the retention times and resolutions of the analytes due to increased competition for the CD cavity. ACN had a greater influence than MeOH reflecting its greater affinity for the CD cavity. The resolution of flavonoids and dansyl amino acids was much better with ACN/ H₂O than that with MeOH/ H₂O on **CSP4**. However, Tröger's base was only resolved with MeOH/ H₂O on **CSP4** and no separation was observed with ACN/ H₂O despite of the longer retention time (Figure 5-6 a). A similar situation was observed for the separation of bendroflumethiazide on **CSP5**. ACN/ H₂O afforded fair resolution while no enantiodiscrimination was observed with MeOH/ H₂O (Figure 5-6 b). ACN was a more powerful eluting solvent than MeOH, but in some cases the H-bonding of MeOH proved to be important [205].

Figure 5-6 Influence of organic modifiers on enantioseparation (a) Tröger's base on **CSP4**; (b) bendroflumethiazide on **CSP5**

5.3 Summary

Two multiple “click” chemistry derived cyclodextrin chiral stationary phases **CSP4** and **CSP5** were successfully employed in reversed phase mode HPLC for the enantioseparation of racemic aryl alcohols, flavonoids, hydroxyl, carboxylic and dansyl amino acids, β -blockers, β -agonists, thiazides and other pharmaceutical chiral compounds.

CSP4 was found amenable for the resolution of hydroxyl acids, carboxylic acids, dansyl amino acids, Tröger's base, indoprofen and ketoprofen whose chiral discrimination cannot be effected on **CSP5**. Both **CSP4** and **CSP5** could separate flavonoids with better resolution on **CSP5**. Aryl alcohols, β -blockers, β -agonists and atropine could only be resolved on phenylcarbamoylated CD based **CSP5**.

Different inclusion mode would take place for different racemic analytes and the hydroxyl groups on CD primary and secondary faces may act differently in H-bond

formation depending on the analytes' structures.

Organic modifiers played important roles in the chiral discrimination process. Most of the analytes could be resolved with either ACN or MeOH while a few discriminations were only available with specific organic solvents.

Chapter 6

Experimental

6.1 Reagents and materials

β -cyclodextrin, calcium hydroxide, methyl iodide, triphenylphosphine, cuprous iodide, copper sulphate, 3-aminopropyltriethoxysilane, 1,1,2,2-tetrachlorethane, ethyl propiolate, propiolic acid, *N,N'*-dicyclohexylcarbodiimide (DCC), sodium ascorbate and iodine were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA) and Fluka (Buchs, Switzerland). Sodium hydroxide, sodium dihydrophosphate, *p*-toluenesulfonyl chloride, imidazole, sodium azide, acetic acid, pyridine and phenyl isocyanate were obtained from Merck (Frankfurter, Darmstadt, Germany). Toluene, acetone, ethyl acetate, hexane, dichloromethane, chloroform, HPLC-grade methanol (MeOH), acetonitrile (ACN), tetrahydrofuran (THF), dimethylformamide (DMF) and triethylamine (TEA) were purchased from Fisher (Fair Lawn, New Jersey, USA). DI and ultra-pure water were prepared with an Arium 611VF water system supplied by Sartorius Stedim Biotech (Goettingen, Germany).

Fused-silica capillaries (100 μm i.d. 365 μm o.d.) were purchased from Deleone Corporation (USA) and stainless steel columns (4.6 μm i.d.) were ordered from Scientific Resources (Singapore). The alkene substituted aryl alcohols were prepared by reducing the corresponding ketone with LiAlH_4 in anhydrous THF and the other analytes were purchased from Sigma-Aldrich and Merck. Kromasil spherical silica gel (5 μm , 100 Å, 300 m^2/g) was purchased from Eka Chemicals (Bohus, Sweden).

General synthetic methods. All the chemicals for organic synthesis were purchased in high purity and used directly without further purification. Pyridine was freshly distilled after refluxing with calcium hydride (CaH_2) for four hours. Toluene was dried by refluxing over sodium wires for 2 hours and used freshly. Anhydrous DMF was prepared by refluxing over CaH_2 under reduced pressure after standing over CaH_2 overnight. β -CD was vacuum-dried (10 mmHg, 60 °C) overnight before use. All the reaction requiring anhydrous conditions were carried out under nitrogen atmosphere using oven-dried glassware.

6.2 Instruments and separation conditions

Fourier transform infrared spectroscopy (FTIR): FTIR spectra were collected on FTS165 FTIR supplied by Perkin-Elmer (Hercules, CA, USA). The measurements were performed at a resolution of 4 cm^{-1} with 32 scans at room temperature. Samples were prepared as KBr pellets.

Elemental analysis (EA): Elemental analysis was performed on Vario EL universal CHNOS elemental analyzer (Elementar Analysensysteme, Hanau, Germany) for C, H and N determinations within $\pm 0.4\%$ of theoretical values.

Nuclear magnetic resonance spectroscopy (NMR): NMR spectra were recorded on a Bruker ACF300 (^1H : 300 MHz; ^{13}C : 75 MHz) supplied by Bruker Biospin (Fällanden, Switzerland). Chemical shifts were reported in parts per million (ppm) by using the δ scale. The following abbreviations were used in reporting spectra, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet.

Mass spectroscopy (MS): MS were obtained on a QSTAR XL LC/MS system purchased from Applied Biosystems (Foster City, CA, USA).

CEC Separation conditions: All CEC separations were performed on a Beckman P/ACE MDQ Capillary Electrophoresis System with diode array detection (DAD) (Fullerton, CA, USA). CEC separation conditions: the mobile phase used was a mixture of acetonitrile and phosphate buffer which was prepared by dissolving NaH_2PO_4 in ultra-pure water (concentration 1.5-13 mM) and adjusted to the desired pH value (pH = 5-8) with 1 M sodium hydroxide. Temperature, detection wavelength and injection mode used were 25 °C, 200-300 nm and 5 kV for 3 s, respectively. Both ends of the capillary column were subjected to pressure at 50 psi. Samples were dissolved in ACN or MeOH/water (40/60 v/v) in the concentration range of 0.2–0.5 mg/mL. All the mobile phases were prepared freshly, filtrated and degassed before use.

HPLC Separation conditions: HPLC enantioseparations were performed on a Hitachi HPLC system equipped with diode array detection (DAD) system (Tokyo, Japan). HPLC separation conditions: the mobile phases were prepared by mixing different amounts of ACN/ MeOH with ultra-pure water or 1% (w/w) triethylammonium acetate buffer (denoted as 1% TEAA, adjusted with acetic acid for the desired pH). All the buffers were filtered through 0.45 µm filter membrane. Detection wavelength was set a range from 200 to 300 nm. The test samples were dissolved in MeOH or ACN/ water (1:1 v/v) at a concentration of 1 mg/mL and the injected volume was 2-4 µL. Each solution was injected in triplicates.

6.3 Calculation method

Both the CEC and HPLC instruments are controlled by 32 Karat system, United States Pharmacopeia (USP) Calculation allows us to track our columns' performance. The parameters are shown in Figure 6-1.

Figure 6-1 Sketch chromatogram of the enantioseparation

The commonly used evaluation equation for the capacity/ retention factor is calculated as:

$$k = \frac{t_R - t_0}{t_0} \dots\dots\dots(1)$$

The USP calculation methods are as follows:

Theoretical Plates

$$n = 16 \left[\frac{t_R}{W} \right]^2 \dots\dots\dots(2)$$

Enantioselectivity

$$\alpha = \frac{k_2}{k_1} \dots\dots\dots(3)$$

Chiral resolution

$$R_s = \frac{2(t_2 - t_1)}{(W_1 + W_2)} \dots\dots\dots(4)$$

Where: n is theoretical plates

t_0 is the dead time (the elution time of the unretained marker or solvent)

t_1 and t_2 are the retention times for the two enantiomers

W is the width of the base of the component peak using tangent method

6.4 Syntheses and CSP preparation

6.4.1 Syntheses

6.4.1.1 Synthesis of mono(6^A-tosyl)- β -CD 2

1-(*p*-tosyl)-imidazole 1

Imidazole (25.5 g, 0.375 mol) and *p*-toluenesulfonyl chloride (75 g, 0.4 mol) were dissolved in dichloromethane (300 mL) in an Erlenmeyer flask. The solution was added with a solution of sodium bicarbonate (39.25 g, 0.47 mol) in water (500 mL) and followed by addition of triethylamine (5.63 mL, 0.055 mol) as phase transfer catalyst. The two layer mixture was stirred at room temperature. Carbon dioxide gas was released during the reaction and the mixture was continuously stirred

overnight. The dichloromethane layer was separated from the aqueous layer, and the aqueous layer was then extracted with dichloromethane. The combined organic layer was dried with anhydrous MgSO_4 , filtered, concentrated to about 125 mL, and added with hexane (25 mL). The mixture was allowed overnight to form white crystal. The crystal was filtered and finally dried under vacuum to afford desired 1-(*p*-tosyl)-imidazole **1**. Yield: 95%.

Characterization [175]: Mp: 77-78 °C; ^1H NMR (CDCl_3) δ (ppm): 2.46 (3H, s, CH_3), 7.10 (1H, s, N-CH=C), 7.31(1H, s, N-CH=C), 7.35(2H, d, H-Ar), 7.83(2H, d, H-Ar), 8.03(1H, s, N-CH=N); ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 21.2, 117.9, 127.4, 130.5, 131.6, 134.9, 136.8, 146.5; FTIR (cm^{-1} , KBr): 3159, 3103, 3032, 1595, 1516, 1383, 1151; ESI-MS (m/z): 223.03 $[\text{M}+\text{H}]^+$, calcd 223.05.

Mono(6^A-tosyl)- β -CD **2**

Dry β -CD (20.0 g, 17.6 mmol) was dissolved in 450 mL water (60 °C) and cooled to room temperature. To the resulting suspension was added finely powdered 1-(*p*-tosyl)-imidazole (15.5 g, 70 mmol) in one portion. After 2 h, a solution of sodium hydroxide (9 g, 0.23 mol) in water (25 mL) was added over 20 min. After 10 min, the reaction was quenched by addition of ammonium chloride (24.1 g, 0.45 mol) with swirling to dissolve all the solids. The resulting mixture was concentrated to about half of its original volume by blowing a stream of air over its surface overnight. The resulting suspension was filtered and the collected solid was washed with two portions (100 mL) of ice water and one portion of acetone (200 mL) and then dried to constant weight over calcium chloride in vacuum to afford

the final product **2**. Yield: 30%.

Characterization [175]: Mp: 165-167 °C (dec); ^1H NMR (DMSO- d_6) δ (ppm): 2.50 (3H, s, CH_3), 3.3-3.6 (overlap with HDO, 42H, m, H-2, H-3, H-4, H-5 and H-6), 4.15-4.2 (1H, m, OH-6), 4.28-4.35 (1H, m, OH-6), 4.41-4.48 (4H, m, OH-6), 4.76 (2H, d, H-1), 4.83 (5H, d, H-1), 5.62-5.84 (14H, m, OH-3 and OH-2), 7.41-7.44 (2H, d, H-Ar), 7.73-7.76 (2H, d, H-Ar); ^{13}C NMR (DMSO- d_6) δ (ppm): 21.3 (CH_3), 59.9, 68.9 (C-6), 69.7, 72.0 (C-5), 72.4 (C-3), 73.0 (C-2), 80.7, 81.5 (C-4), 101.9, 102.1 (C-1), 127.6, 129.9, 132.7, 144.8 (C-Ar). FTIR (cm^{-1} , KBr): 3400, 2935, 1647, 1367, 1159, 1080, 1031, 582; ESI-MS (m/z): 1311.2 $[\text{M}+\text{H}]^+$, calcd 1311.6.

6.4.1.2 Synthesis of mono(6^A-azido-6^A-deoxy)- β -CD **3**

TsO- β -CD (2.5 g, 2.0 mmol) and sodium azide (1.95 g, 30 mmol) were dissolved in DMF (20 mL). The solution was heated to 90 °C for 2 days, after which the solution was evaporated to half of its volume and 1,1,2,2-tetrachloroethane (5 mL) was added to form a precipitates. The resultant mixture was stirred for 1 h. The complex formed was separated and then dissolved in boiling water to afford N₃- β -CD. Yield: 80%.

Characterization [119]: Mp: 208 °C (dec); ^1H NMR (DMSO- d_6) δ (ppm): 3.32-3.40 (28H, m, H-3, H-5 and H-6), 3.55-3.64 (14H, m, H-2 and H-4), 4.44-4.54 (6H, m, OH-6), 4.83-4.87 (7H, br s, H-1), 5.62-5.77 (14H, m, OH-2 and OH-3); ^{13}C NMR (DMSO- d_6) δ (ppm): 51.1, 60.3 (C-6), 70.8, 72.1 (C-5), 73.1 (C-3), 73.3 (C-2),

81.3, 82.3 (C-4), 101.9, 102.1 (C-1); FTIR(cm^{-1} , KBr): 2104 ($-\text{N}_3$ str); ESI-MS (m/z): 1182.5 $[\text{M}+\text{Na}]^+$, calcd 1182.

6.4.1.3 Synthesis of mono(6^A-azido-6^A-deoxy)-permethylated- β -CD 4a

Dry N_3 - β -CD (2.0 g, 1.7 mmol) was dissolved in dried DMF (30 mL). The dissolved N_3 - β -CD solution was added dropwise to the sodium hydride (2.0 g, 84.0 mmol) which was previously washed with hexane under N_2 to remove paraffin. After the gas evolution ceased, methyl iodide (5 mL, 35.0 mmol) was added slowly to the reaction mixture and stirred for 12 h. Excess of water was then added, the resulting mixture was extracted with ethyl acetate (3×50 mL) thrice. The combined organic layer was washed with water (3×50 mL), dried over anhydrous magnesium sulfate and evaporated under vacuum. The crude product was then purified by chromatography using ethyl acetate/acetone (1:1, v/v) as the eluent to afford Me- N_3 -CD. Yield: 78%.

Characterization [119]: Mp: 96-99 $^{\circ}\text{C}$; ^1H NMR (CDCl_3) δ (ppm): 3.15-3.24 (7H, m, H-5), 3.40 (18H, s, OCH_3), 3.50-3.95 (77H, m, H-2, H-3, H-4, H-6 and OCH_3), 5.05 (1H, d, H-1^A), 5.13 (6H, d, H-1); ^{13}C NMR (CDCl_3) δ (ppm): 52.0, 58.4, 58.6 (2- CH_3), 58.8 (6- CH_3), 61.2, 61.3, 61.4 (3- CH_3), 70.7, 70.8, 71.0 (C-5), 71.2, 71.3, 71.5 (C-6), 79.9, 80.0, 80.2, 88.3 (C-4), 81.3, 81.7 (C-2), 81.8, 82.0 (C-3), 98.3, 98.8, 99.0, 99.2 (C-1); FTIR (cm^{-1} , KBr): 2930, 2104 ($-\text{N}_3$ str), 1030; ESI-MS (m/z): 1462.7 $[\text{M}+\text{Na}]^+$, calcd 1462.4; Anal. Calcd ($\text{C}_{62}\text{H}_{109}\text{N}_3\text{O}_{34}$): C 51.7%, H

7.62%, N 2.92% Found: C 52.1%, H 7.97%, N 2.56%.

6.4.1.4 Synthesis of mono(6^A-azido-6^A-deoxy)-perphenylcarbamoylated- β -CD

4b

Phenyl isocyanate (13.44 g, 110.4 mmol) was added to a solution of N₃- β -CD (2.23 g, 1.92 mmol) in 35 mL of dry pyridine (reluxed for 4 h over CaH₂), the mixture was stirred overnight at 85 °C. The solvent was removed by distillation under vacuum to give a yellow brown gel. The gel was dissolved in ethyl acetate (140 mL) and stirred for about 2 h after H₂O (60 mL) was added. Filtration was applied and the organic layer of the filtrate was dried on anhydrous magnesium sulfate. Crude product obtained after removing the solvent was purified by chromatography using ethyl acetate-hexane (1:2, v/v) as mobile phase to afford Ph-N₃-CD. Yield: 49%.

Characterization [119]: Mp: 225-227 °C; ¹H NMR (CDCl₃) δ (ppm): 3.98 (7H, m, H-4), 4.44-4.63 (21H, m, H-1, H-5 and H-6), 5.15-5.22 (14H, m, H-2), 5.59 (7H, m, H-3), 6.88-7.45 (120H, m, NHCO, H-Ar); ¹³C NMR (CDCl₃) δ (ppm): 54.0, 60.1 (C-6), 69.5-69.8 (C-2), 70.4-70.7 (C-5), 72.3 (C-3), 98.4 (C-1), 118.0-128.2 (C-Ar); FTIR (cm⁻¹, KBr): 3396, 3317, 2106 (-N₃ str), 1739, 1602, 1500; ESI-MS (m/z): 3676.2 [M+K]⁺, calcd 3677.1; Anal. Calcd (C₁₈₇H₁₇₅N₂₃O₅₆): C 61.7%, H 4.65%, N 8.85% Found: C 61.3%, H 4.68%, N 8.85%.

6.4.1.5 Synthesis of heptakis(6-azido-6-deoxy)-2,3-di-O-β-CD (heptakis-N₃-CD)

6

Pre-dried β-CD (2 g) was added to a solution of iodine (10 g) and triphenylphosphine (10 g) in dry DMF (40 mL). The reaction mixture was stirred at 90 °C for 12 h. After reaction, excess DMF (about 20 mL) was removed under vacuum and the pH of the reaction system was adjusted to 9~10 with 3 M NaOCH₃. The precipitated product was collected by filtration and washed with acetone to afford iodinated β-CD **5** with a yield of 60%. Thereafter, **5** was dissolved in 50 mL DMF and reacted with sodium azide (2.8 g) under 90 °C overnight. DMF was removed and the reaction mixture was poured into cold water and filtrated. The obtained solid was then washed with water and vacuum dried to afford heptakis(6-azido-2,3-di-O)-β-CD **6**. Yield: 80%.

Characterization [120]: Mp: 220 °C (dec); ¹H NMR (DMSO-d₆) δ (ppm): 3.5-4.0 (42H, H-2, H-3, H-4, H-5 and H-6), 4.9-5.0 (7H, s, H-1), 5.6-5.9 (14H, m, OH-2 and OH-3); ¹³C NMR (DMSO-d₆) δ (ppm): 70.5, 71.4, 73.9, 76.7, 79.3, 100.5; FTIR(cm⁻¹, KBr): 3396, 2928, 2106 (N₃ str), 1051; ESI-MS (m/z): 1331.4 [M+Na]⁺, calcd 1332.

6.4.1.6 Synthesis of heptakis(6-azido-6-deoxy)-2,3-di-O-phenylcarbamoylated- β -CD 7

Phenyl isocyanate (10 mL) was added to the solution of **6** (2.0 g) in dried pyridine (30 mL dried over CaH_2). The reaction mixture was heated to 90 °C and kept for 12 h protected with N_2 . After removal of pyridine and the unreacted phenyl isocyanate under vacuum, the residue was dissolved in ethyl acetate (100 mL) and water (50 mL) was added with vigorous stirring. The organic layer was separated, washed with water (2×50 mL), and dried on anhydrous magnesium sulphate. After removal of the solvent, the resulting residue was purified by flash chromatography using hexane-ethyl acetate (2:1, v/v) as the eluting phase to afford light yellow solid **7**. Yield: 65%.

Characterization [120]: Mp: 202-205 °C; ^1H NMR (CDCl_3) δ (ppm): 3.8-5.6 (49H, m, H-1, 2, 3, 4, 5 and 6), 6.8-7.5 (84H, NH and H-Ar); ^{13}C NMR (CDCl_3) δ (ppm): 61.1 (C-6), 69.6-69.9 (C-2), 71.4-71.7 (C-5), 78.2 (C-3), 98.2 (C-1), 117.1-126.2 (C-Ar); FTIR (cm^{-1} , KBr): 3395, 3313 (N-H str), 3060, 2106 ($-\text{N}_3$ str), 1744, 1602, 1532, 1445, 1054; ESI-MS (m/z): 2997.3 $[\text{M}+\text{Na}]^+$, calcd. 2998.

6.4.1.7 Synthesis of N-3-(triethoxysilyl)propyl-2-propynamide 8

To a solution of 3-aminopropyltriethoxysilane (2.4 g, 10.85 mmol) and propiolic

acid (0.87 g, 11.93 mmol) in dichloromethane (15 mL) was added *N,N'*-dicyclohexylcarbodiimide (2.46 g, 12.0 mmol). The reaction mixture was kept at room temperature for 1 h and then filtered. Evaporation of the solvent was followed by coevaporation with toluene (2 × 50 mL) to afford the final liquid product **8**. Yield: 80%.

Characterization: ^1H NMR (CDCl_3) δ (ppm): 0.56-0.61 (2H, t, CH_2Si), 1.14-1.19 (9H, t, OCH_2CH_3), 1.58-1.63 (2H), 2.28 (1H, s, $\text{C}\equiv\text{CH}$), 3.21-3.27 (2H, m, CH_2N), 3.72-3.79 (6H, q, CH_2O), 7.11 (1H, m, NH); ^{13}C NMR (CDCl_3) δ (ppm): 7.6, 18.3, 22.3, 43.4, 58.4, 73.3, 77.9, 163.2; FTIR(cm^{-1} , KBr): 2110 (alkyne str); ESI-MS (m/z): 296.1 $[\text{M}+\text{Na}]^+$, calcd 296.

6.4.1.8 Synthesis of cuprous iodide-triphenylphosphine complex $\text{CuI}(\text{PPh}_3)$ **9**

A solution of triphenylphosphine (0.69 g, 2.63 mmol) in acetonitrile (10 mL) was added to a solution of CuI (0.5 g, 2.63 mmol) in the same solvent 50 mL under N_2 . After complex precipitation, the mixture was stirred for 1 h and filtered. The collected solid was washed with acetonitrile and vacuum-dried to afford the soluble catalyst **9**. Yield: 90%.

Characterization: FTIR (cm^{-1} , KBr): 1479, 1434, 1097, 748, 695, 521, 503. Anal. Calcd ($\text{C}_{18}\text{H}_{15}\text{PCuI}$): C 47.6 %, H 3.31% Found: C 47.2%, H 3.29%.

6.4.1.9 Preparation of alkynyl functionalized silica 10

Activated 5 μm silica gel (160 $^{\circ}\text{C}$, 24 h under vacuum) was suspended in dried toluene (30 mL) and then the synthesized N-3-(triethoxysilyl)propyl-2-propynamide **8** was added. The reaction mixture was heated under reflux for 2 h. The resultant mixture was evaporated up to half of its original volume followed by further addition of dry toluene (15 mL) and refluxed for another 2 h. After that, the reaction mixture was filtered and the solid was washed with dichloromethane, then extracted with acetone for 24 h before vacuum-dried to afford alkynyl functionalized silica **10**.

Characterization: FTIR(cm^{-1} , KBr): 2121 ($-\text{C}\equiv\text{CH}$); Anal.found: C 5.32%, H 0.986%, N 0.846%.

6.4.1.10 Synthesis of 1-(6^A-deoxy- β -CD)-4-(ethoxycarbonyl)-1,2,3-triazole 11

To a solution of ethyl propiolate (100 mg, 1.74 mmol) in DMF (15 mL) was added N₃- β -CD **3** (2 g, 1.74 mmol) and anhydrous copper sulfate (278.4 mg, 1.74 mmol). Freshly prepared sodium ascorbate (696 mg, 3.48 mmol) in aqueous DMF (50 %) was added drop-wise and the reaction was stirred for 48 h at room temperature. After this period the mixture was filtered, and the solvent was removed under

vacuum. The residue was then dissolved in 10 mL DMF, filtered and the solvent was again evaporated. Flash chromatography (silica, 30:70 ACN/H₂O) afforded 1.7 g of **11** as a white solid. Yield: 80%.

Characterization: ¹H NMR (DMSO-d₆) δ (ppm): 1.27-1.31 (3H, t, CH₃), 3.23-3.79 (42H, m, H-2, 3, 4, 5 and 6), 4.26-4.30 (2H, q, CH₂O), 4.45-4.53 (6H, m, OH-6), 4.75 (7H, s, H-1), 5.64-5.93 (14H, OH-2 and OH-3), 8.72 (1H, s, C=CHN); ¹³C NMR (DMSO-d₆) δ (ppm): 12.9, 60.6, 63.3, 72.7, 73.2, 73.4, 81.3, 82.4, 101.8, 102.1, 131.5 156.0; FTIR (cm⁻¹, KBr): 3415, 1724, 1230, 1031; ESI-MS(m/z): 1280.4 [M+Na]⁺, calcd 1281; Anal. Calcd (C₄₇H₇₅N₃O₃₆): C 44.9%, H 6.01%, N 3.34% Found: C 44.5%, H 6.21%, N 3.29%.

6.4.1.11 Synthesis of 1-(6^A-deoxy-permethylated-β-CD)-4-(ethoxycarbonyl)-1,2,3- triazole **12**

To a solution of ethyl propiolate (40 mg, 0.7 mmol) in DMF (6 mL) was added N₃-Me-CD **3** (1 g, 0.7 mmol) and anhydrous copper sulfate (112.2 mg, 0.7 mmol). Freshly prepared sodium ascorbate (279 mg, 1.39 mmol) in aqueous DMF (50 %) was added drop-wise and the reaction was stirred for 48 h at room temperature. After this period the mixture was filtered, and the solvent was removed under vacuum. The residue was then dissolved in 5 mL DMF, filtered and the solvent again evaporated. Flash chromatography purification afforded 0.81 g of **13** as a

light yellow solid. Yield: 80%.

Characterization: ^1H NMR (CDCl_3) δ (ppm): 1.25-1.32 (3H, t, CH_3) 3.21-4.05 (101H, m, H-2, 3, 4, 5, 6 and OCH_3), 4.12-4.19 (2H, q, CH_2O), 4.92 (7H, s, H-1), 8.21 (1H, s, $\text{C}=\text{CHN}$); ^{13}C NMR (CDCl_3) δ (ppm): 12.8, 29.6, 58.7, 58.9, 61.1-61.4, 70.7-71.5, 78.9, 81.2, 97.3-91.2 121.9, 148.2. FTIR (cm^{-1} , KBr): 2930, 1741, 1030; ESI-MS (m/z): 1560.1 $[\text{M}+\text{Na}]^+$, calcd 1560.4; Anal. Calcd ($\text{C}_{67}\text{H}_{115}\text{N}_3\text{O}_{36}$): C 52.3%, H 7.53%, N 2.73% Found: C 52.1%, H 7.57%, N 2.71%.

6.4.1.12 Synthesis of 1-(6^A-deoxy-perphenylcarbamoylated- β -CD)-4-(ethoxycarbonyl)-1,2,3-triazole **13**

To a solution of ethyl propiolate (16.3 mg, 0.28 mmol) in DMF (6 mL) was added N_3 -Ph-CD **4b** (1 g, 0.28 mmol) and anhydrous copper sulfate (44.8 mg, 0.28 mmol). Freshly prepared sodium ascorbate (91 mg, 3.48 mmol) in aqueous DMF (50 %) was added drop-wise and the reaction was stirred for 48 h at room temperature. After this period the mixture was filtered, and the solvent was removed under vacuum. The residue was then dissolved in ethyl acetate and washed with saturated NaCl solution. The organic layer was concentrated and the collected solid was purified by flash chromatography with ethyl acetate/hexane as the eluent to afford **13** as a light-yellow solid. Yield: 70%.

Characterization: ^1H NMR (CDCl_3) δ (ppm): 1.26-1.30 (3H, t, CH_3), 3.99-5.62 (51 H, m, H-1, 2, 3, 4, 5 and 6), 6.88-7.45 (120H, m, NH and H-Ar), 8.55 (1H, s, $\text{C}=\text{CHN}$); ^{13}C NMR (CDCl_3) δ (ppm): 14.3, 32.1, 53.6, 62.1, 69.5-69.8, 70.2-70.6, 72.5, 98.7, 118.0-128.2 (C-Ar), 122.3, 153.1 ; FTIR (cm^{-1} , KBr): 3396, 3317, 1739, 1602, 1500; ESI-MS(m/z): 3657.8 $[\text{M}+\text{Na}]^+$, calcd 3658; Anal. Calcd ($\text{C}_{47}\text{H}_{75}\text{N}_3\text{O}_{36}$): C 44.9 %, H 6.01%, N 3.34% Found: C 44.5%, H 6.21%, N 3.29%.

6.4.2 “Click” chemistry preparation of CSPs

General procedure: Alkynyl functionalized silica **10** (0.5 g) was suspended in a solution of β -CD derivatives (**3**, **4**, **6**, **7** in proper amount) in DMF (5 mL) and then $\text{CuI}(\text{PPh}_3)$ (10 mmol %) was added. The reaction mixture was heated to 80 °C and kept for two days. The crude products were washed with DMF and thereafter extracted with acetone/methanol for 24 h before being vacuum dried to afford the final **CSP1** to **5**.

Characterization: **CSP1**, FTIR (cm^{-1} , KBr): 2940, 3400. Anal.found: C 14.35%, H 2.639%, N 1.49%. **CSP2**, FTIR (cm^{-1} , KBr): 2933. Anal.found: C 16.62%, H 2.616%, N 1.63%; **CSP3**, FTIR (cm^{-1} , KBr): 1725, 1448. Anal.found: C 12.49%, H 1.794%, N 1.976%; **CSP4**, FTIR (cm^{-1} , KBr): 2943, 3470. Anal.found: C 11.31%, H 2.26%, N 3.90%; **CSP5**, FTIR (cm^{-1} , KBr): 1738, 1448. Anal.found: C 13.57%, H 1.89%, N 3.18%;

6.5 Column preparation

6.5.1 Capillary column preparation

The CEC column used in this work was 29 cm long with an effective length of 9 cm. Before packing, the detection window was first made by burning off a small section of polyimide. The capillary was then flushed with methanol for about 30 minutes followed by blow with nitrogen for 20 minutes. To create a frit, the end near to the detection window was dipped into a concentrated bare silica/water suspension and subsequently fused by burning with a capillary burner. A slurry consisted of 10 mg/mL of packing material in methanol was sonicated for 5 minutes and delivered to the pressure cell. Packing procedure was carried out at 5000 psi using HPLC pump (LabAlliance-Scientific, USA). After packing, the pump was turned off to allow the pressure dropping slowly. The column was then flushed with water for 1 hour at 5000 psi followed by sintering of the inlet and outlet frits. The retain frit was cut off and the column was flushed reversely to remove the undesired material.

6.5.2 Preparation of HPLC column

The prepared CSPs were packed by slurry technique with methanol as the solvent. The CSP slurry was packed into a stainless steel column (ϕ 4.6 mm $\times$ 15 cm) at 8000 psi for 30 min before the pressure was released slowly. The steel column was removed from the HPLC pump and sealed for use.

Chapter 7

Conclusions

A novel preparation of cyclodextrin grafted chiral stationary phases (CSPs) has been developed based on Cu (I) catalytic 1,3-dipolar cycloaddition (“click” chemistry) and examined in this dissertation. Five functionalized azido CD derivatives were synthesized and anchored onto alkynyl modified silica surfaces via single and multiple triazolyl linkages. Enantioseparation of various racemic compounds on capillary electrochromatography (CEC) and high performance liquid chromatography (HPLC) using the newly derived CSPs have been successfully carried out. The influences of CEC and HPLC parameters as well as the CSP structures on the enantioseparation were also investigated.

In order to achieve “click” immobilization of CDs, different azido CD derivatives including N₃-β-CD, N₃-Me-CD, N₃-Ph-CD, heptakis-N₃-β-CD and heptakis-N₃-Ph-CD were synthesized and alkynyl groups were introduced to silica surface by reaction of chemically synthesized N-3-(triethoxysilyl)propyl-2-propynamide on silica in toluene. Prior to surface immobilization of chiral selectors, “click” reactions using model compounds were performed by reaction of CD azido groups with the alkynyl moiety of ethyl propiolate catalyzed by copper sulphate and sodium ascorbate, affording three novel CD-based triazoles (ETCD, ETMeCD and ETPhCD). It was found that the use of copper sulphate and sodium ascorbate would afford Cu (0) particles during the reaction, which was not suitable for heterogeneous reactions on silica surface. In order to improve catalyst performance and simplify CSP purification, a Cu (I) complex [CuI(PPh₃)] which was soluble in organic solvents was synthesized from

cuprous iodide and triphenylphosphine and used as the catalyst for “click” immobilization. Three structurally well-defined CD-based CSPs (**CSP1**, **CSP2** and **CSP3**) were prepared via single triazolyl linkage and two other stable CSPs (**CSP4** and **CSP5**) via multiple triazolyl linkages. High surface loadings of 0.59, 0.51, 0.11, 0.35 and 0.10 $\mu\text{mol}\cdot\text{m}^{-2}$ for **CSP1** to **5** respectively revealed that reliability of this facile approach to the preparation of cyclodextrin based chiral stationary phases. In addition, “click” chemistry could afford native CD derived CSPs which was not available via Staudinger reaction reported previously [119].

Three new chromatographic chiral capillary columns were successfully fabricated by packing the single triazolyl linkage derived CSPs (**CSP1**, **CSP2** and **CSP3**) into 100 μm i.d. fused silica capillaries with sintered frits. Highly efficient (23000-25000 plates/m using thiourea probe) “pure” capillary electrochromatography was achieved by solely exploiting the electroosmotic flow (EOF) for efficient separation. Good stability of the packed capillaries was attested to by the linear relationship between the EOF and applied voltage. A series of neutral racemic aryl alcohols and flavonoids have been efficiently separated into their enantiomers on the three newly packed capillaries. The influences of buffer pH, applied voltage, buffer concentration and acetonitrile content on the enantioseparation were examined on **CSP3** packed capillary using a model aryl alcohol. It was found that, for neutral analytes, (i) the applied voltage and organic modifier played important roles in CEC separation; (ii) altering buffer pH only slightly influenced the EOF and resolution; (iii) decreasing buffer concentration

could lead to peak band-broadening; (iv) higher concentration resulted in greater Joule heating which could affect the resolution. Optimum separation conditions for aryl alcohols were found with 5 mM NaH₂PO₄ buffered at pH=7, 40% acetonitrile as the mobile phase and 5 kV as the applied voltage. Apart from the high efficiency and resolution of CEC, some drawbacks such as difficulty in capillary fabrication and storage as well as short shelf life were also found with this technique.

The enantioseparation abilities of the obtained **CSP1** to **5** were broadly investigated in reversed phase mode HPLC by enantioseparation of a variety of racemic compounds including dansyl amino acids, hydroxyl and carboxylic acids, flavanoids, fragrance ionones, aryl alcohols, β -blockers, β -agonists, thiazides, nimodipine, brompheniramine, Tröger's base, chlorthalidone, atropine, benzoin and *p*-chlorophenyl-pyrimidine. The influences of CSP structures, mobile phase compositions and analytes' structures on the enantioseparation were investigated in detail. The following points can be summarized from the series of elaborate investigations of the derived CSPs in reversed phase mode HPLC:

- a) Functional groups on the CD rims strongly affected the enantiomeric separation process. Different functional groups rendered the corresponding CSPs with diverse enantiorecognition abilities. In this dissertation, native CD **CSP1** and **CSP4** could resolve dansyl amino acids, hydroxyl and carboxylic acids, flavanone and hydroxyl flavanones, nimodipine, chlorthalidone and Tröger's base well. Methylated CD **CSP2** afforded the best separations of the ionones fragrance compounds and partially resolved methoxyflavanones,

while phenylcarbamoylated CD **CSP3** and **CSP5** showed the most powerful enantiorecognition ability towards racemic aryl alcohols, β -blockers, β -agonists, atropine and *p*-chlorophenyl-pyrimidine. The broader enantiorecognition abilities of **CSP1** and **CSP3** over **CSP2** were attributable to the hydrogen bonding sites (-OH on **CSP1**, N-H on **CSP3**) and π - π interaction sites (phenyl rings on **CSP3**), which strongly favored the CD chiral recognition ability.

- b) Generally, CSP with single triazolyl linkage exhibited better chiral recognition abilities than that with multiple triazolyl linkages. Thus, **CSP1** which has single linkage affords fairly better enantiodiscrimination to racemic dansyl amino acids, flavanone and hydroxyl flavanones, nimodipine and chlothalidone in comparison to **CSP4** which has multiple linkages. However, **CSP4** could better resolve racemic hydroxyl and carboxylic acids. Furthermore, ketoprofen, indoprofen and 5-methoxyflavanone were solely resolved only on **CSP4**. Different inclusion modes have been proposed to explain these diversities for different chiral analytes.
- c) Inclusion complexation between CD cavity and analytes played an important role in sample retention. Generally, increasing organic modifier content would reduce the retention due to its competition with the analyte in occupying the CD hydrophobic cavity. Acetonitrile is a stronger eluting solvent than methanol in reversed phase mode attributed to its stronger affinity with the CD cavity while methanol being a protic solvent behaves differently from

acetonitrile for some cases. Thus, for native CD **CSP1**, acetonitrile was better than methanol whereas the reversed was found for **CSP2** and **CSP3** for most studied analytes. Even for the same CSPs, some specific analytes could only be separated with one organic modifier. For example, Tröger's was well resolved on **CSP1** using H₂O and methanol as the eluting phase, while no separation was found by changing methanol to acetonitrile.

Future work suggestion

As we have determined earlier, the “click” chemistry derived cyclodextrin CSPs have proven to be efficient chiral stationary phases for a broad range of racemic compounds with excellent enantioseparation abilities in high performance liquid chromatography and capillary electrochromatography. This makes them amenable for further utilization in preparative-scale separation in industry and in supercritical fluid chromatography. In addition, in order to judge the commercial potential of the new CSPs, comparison between the new CSP packed columns and the commercial β -CD columns such as CyclobondTM would have been interesting.

In this dissertation, “click” chemistry was only used for immobilization of β -cyclodextrins, this CSP family can be enlarged by further “click” immobilization of α - and γ -cyclodextrin derivatives onto silica beads. In addition, the alteration of spacer arm length between the silica and the CD molecule can be conducted to study the influence of the triazolyl linkage on the enantioseparation process. Although we tried to give some fragmentary explanation for the different enantioselectivities

on the derived CSPs, the perfect mechanisms are still in dark and need further investigation by employing molecular modeling studies.

Ultrahigh performance liquid chromatography (UHPLC) using sub-2 μm silica beads, as a fast and efficient separation technique, has been widely used for achiral separation in recent years. However, there are very few papers reporting enantiomeric separations on UHPLC. The facile “click” reaction may provide a good approach for the efficient immobilization of cyclodextrins onto silica beads with smaller size affording sub-micron chiral stationary phases for high speed chiral analysis in UHPLC.

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