Universidad de
COMISIÓN DE ESTUDIOS OFICIALES
DE POSGRADO Y DOCTORADO

ACTA DE EVALUACIÓN DE LA TESIS DOCTORAL

(FOR EVALUATION OF THE ACT DOCTORAL THESIS)

Año académico (*academic year*): 2019/20

DOCTORANDO (*candidatePHD*): **XU, DONGSHENG**
D.N.I./PASAPORTE (*Id.Passport*): ******24075**
PROGRAMA DE DOCTORADO (*Academic Committee of the Programme*): **D446-QUIMICA**
DPTO. COORDINADOR DEL PROGRAMA (*Department*): **QUÍMICA ANALÍTICA, QUÍMICA FÍSICA E INGENIERÍA QUÍMICA**
TITULACIÓN DE DOCTOR EN (*Phd title*): **DOCTOR/A POR LA UNIVERSIDAD DE ALCALÁ**

En el día de hoy 18/12/19, reunido el tribunal de evaluación, constituido por los miembros que suscriben el presente Acta, el aspirante defendió su Tesis Doctoral **con Mención Internacional** (*In today assessment met the court, consisting of the members who signed this Act, the candidate defended his doctoral thesis with mention as International Doctorate),* elaborada bajo la dirección de (*prepared under the direction of)* MARÍA LUISA MARINA ALEGRE // ZHENGJIN JIANG.

Sobre el siguiente tema *(Title of the doctoral thesis)*: *NEW MONOLITHIC CHIRAL STATIONARY PHASES FOR ENANTIOSEPARATION USING NANO-LC: PREPARATION AND APPLICATIONS*

Finalizada la defensa y discusión de la tesis, el tribunal acordó otorgar la CALIFICACIÓN GLOBAL[1] de (**no apto, aprobado, notable** y **sobresaliente)** *(After the defense and defense of the thesis, the court agreed to grant the GLOBAL RATING (fail, pass, good and excellent)*: Excellent

Alcalá de Henares, a 13 de December de 2019

Fdo. *(Signed)*: Damián Pérez Quintanilla (Secretary)
Fdo. *(Signed)*: ALBERDI (President)
Fdo. *(Signed)*: C. ROMMEN (VOCAL)

FIRMA DEL ALUMNO (candidate's signature),

Fdo. *(Signed)*: Dongsheng Xu

Con fecha 20 de enero de 2020 la Comisión Delegada de la Comisión de Estudios Oficiales de Posgrado, a la vista de los votos emitidos de manera anónima por el tribunal que ha juzgado la tesis, resuelve:

☒ Conceder la Mención de "Cum Laude"
■ No conceder la Mención de "Cum Laude"

La Secretaria de la Comisión Delegada

[1] La calificación podrá ser "**no apto**" "**aprobado**" "**notable**" y "**sobresaliente**". El tribunal podrá otorgar la mención de "cum laude" si la calificación global es de sobresaliente y se emite en tal sentido el voto secreto positivo por unanimidad. (*The grade may be "**fail**" "**pass**" "**good**" or "**excellent**". The panel may confer the distinction of "cum laude" if the overall grade is "Excellent" and has been awarded unanimously as such after secret voting.).*

INCIDENCIAS / OBSERVACIONES:
(Incidents / Comments)

Universidad de Alcalá

ESCUELA DE DOCTORADO

escuela.doctorado@uah.es

DILIGENCIA DE DEPÓSITO DE TESIS.

Comprobado que el expediente académico de D./Dª Dongsheng Xu reúne los requisitos exigidos para la presentación de la Tesis, de acuerdo a la normativa vigente, se procede, con fecha de hoy 270, a registrar el depósito de la tesis en el Servicio de Estudios Oficiales de Posgrado, con el nº de páginas: _____.

Alcalá de Henares a _____ de _____ de 20_____

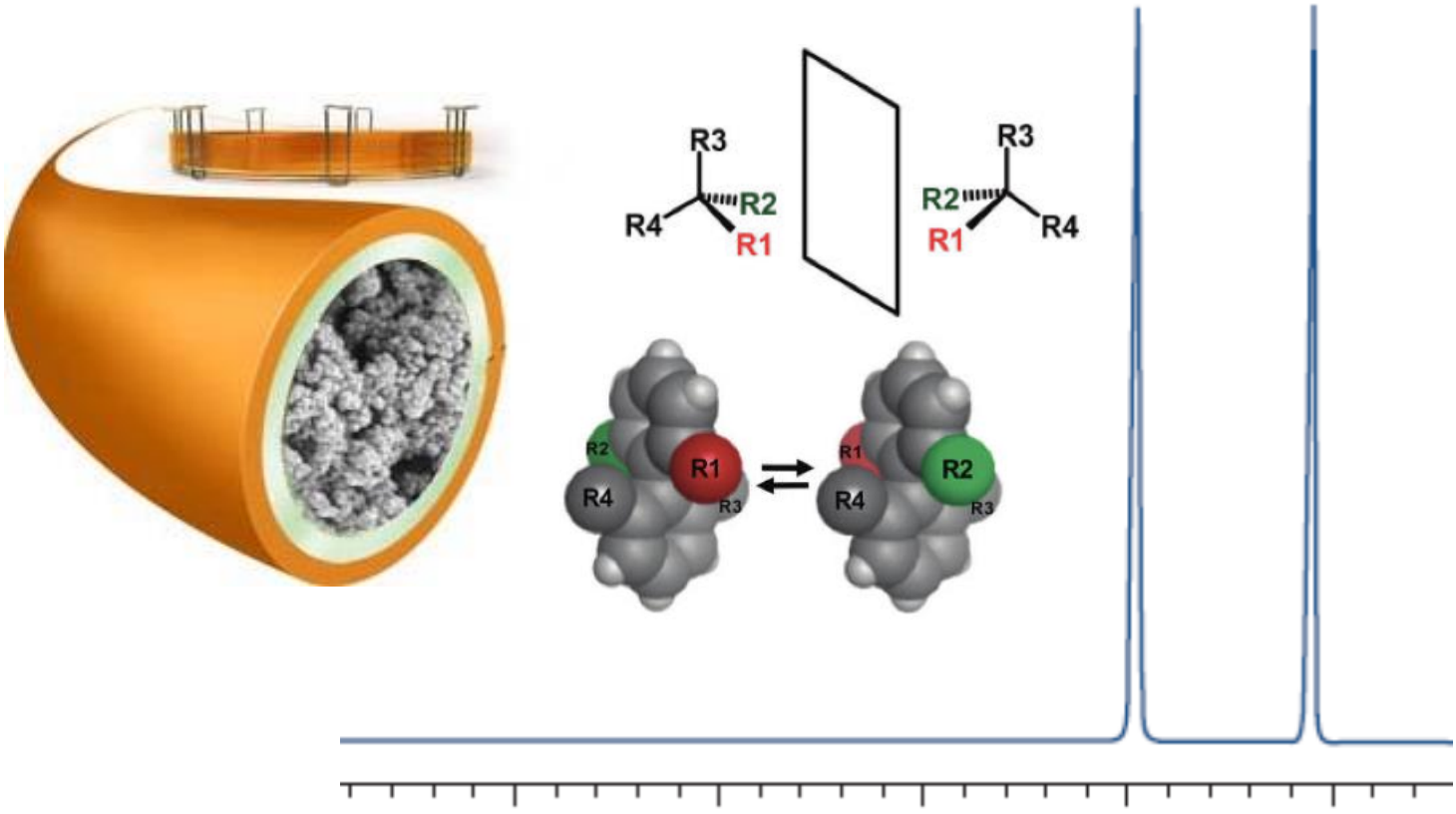
UNIVERSIDAD DE ALCALÁ SERVICIO DE ESTUDIOS OFICIALES DE POSGRADO

Fdo. El Funcionario

Universidad de Alcalá

Programa de Doctorado en Química

NEW MONOLITHIC CHIRAL STATIONARY PHASES FOR ENANTIOSEPARATION USING NANO-LC: PREPARATION AND APPLICATIONS



PhD THESIS BY: DONGSHENG XU

Alcalá de Henares, October 2019

FACULTAD DE CIENCIAS

Departamento de Química Analítica,
Química Física e Ingeniería Química

NEW MONOLITHIC CHIRAL STATIONARY PHASES FOR ENANTIOSEPARATION USING NANO-LC: PREPARATION AND APPLICATIONS

PhD THESIS BY:

DONGSHENG XU

SUPERVISORS:

Prof. MARÍA LUISA MARINA ALEGRE

Prof. ZHENGJIN JIANG

Alcalá de Henares, October 2019

Universidad de Alcalá

Departamento de Química Analítica, Química Física e Ingeniería Química
Facultad de Ciencias
Edificio Polivalente
Ctra. Madrid-Barcelona Km. 33.600
E-28871 Alcalá de Henares (Madrid)

ALBERTO ESCARPA MIGUEL, Coordinador del Programa de Doctorado en Química de la Universidad de Alcalá,

CERTIFICA:

Que el trabajo descrito en la presente memoria, titulado:

"**New monolithic chiral stationary phases for enantioseparation using nano-LC: Preparation and applications**",

ha sido realizado por **D. Dongsheng Xu**, bajo la dirección de los **Dres. María Luisa Marina Alegre y Zhengjin Jiang**, en el Departamento de Química Analítica, Química Física e Ingeniería Química de la Universidad de Alcalá, y en el Institute of Pharmaceutical Analysis, College of Pharmacy, Jinan University. Esta memoria se presenta como compendio de artículos, reuniendo los requisitos exigidos a este tipo de Tesis Doctoral, así como los requisitos científicos de originalidad y rigor metodológicos para ser defendida ante un tribunal. Esta Comisión ha tenido también en cuenta la evaluación positiva anual del doctorando, habiendo obtenido las correspondientes competencias establecidas en el Programa.

Para que así conste a los efectos del depósito de la tesis, se firma en Alcalá de Henares a 29 de octubre de 2019.

UNIVERSIDAD DE ALCALÁ · DEPARTAMENTO QUÍMICA ANALÍTICA, QUÍMICA FÍSICA E INGENIERÍA QUÍMICA ·

Alberto Escarpa Miguel

Universidad de Alcalá

Departamento de Química Analítica, Química Física e Ingeniería Química
Facultad de Ciencias
Edificio Polivalente
Ctra. Madrid-Barcelona Km. 33.600
E-28871 Alcalá de Henares (Madrid)

MARÍA LUISA MARINA ALEGRE, Catedrática de Química Analítica del Departamento de Química Analítica, Química Física e Ingeniería Química de la Universidad de Alcalá, y

ZHENGJIN JIANG, Professor of Pharmaceutical Analysis en el College of Pharmacy de la Universidad de Jinan,

CERTIFICAN:

Que el trabajo descrito en la presente memoria, titulado:

"New monolithic chiral stationary phases for enantioseparation using nano-LC: Preparation and applications",

ha sido realizado bajo su dirección por **D. Dongsheng Xu**, en el Departamento de Química Analítica, Química Física e Ingeniería Química de la Universidad de Alcalá y en el Institute of Pharmaceutical Analysis, College of Pharmacy, Jinan University. Esta memoria se presenta como compendio de cuatro artículos en los que el doctorando es el primer autor. Asimismo, autorizan su presentación para que sea defendido como Tesis Doctoral.

Y para que conste y surta los efectos oportunos, firman el presente en Alcalá de Henares a 25 de octubre de 2019.

María Luisa Marina Alegre **Zhengjin Jiang**

The research work included in this PhD thesis was supported by the Ministry of Economy and Competitiveness (Spain) (projects CTQ2013-48740-P and CTQ2016-76368-P), the Comunidad of Madrid (Spain) and European funding from FEDER program (project S2013/ABI-3028 (AVANSECAL-CM)), the Science and Technology Planning Project of Guangdong Province, China (2016A040403056 and 2015A020211018) and the International Science & Technology Cooperation Program of Guangzhou, China (201807010022).

RESUMEN

Las separaciones quirales constituyen un tema de gran interés en la actualidad debido a su alto impacto en diferentes campos como el farmacéutico, agroquímico o alimentario, entre otros. Aunque existen numerosas fases estacionarias quirales (CSPs) que se encuentran disponibles comercialmente y que incluyen las basadas en ciclodextrinas, polisacáridos, derivados de 3,5-dinitrobenzamida-naftilglicina, proteínas, antibióticos macrocíclicos o alcaloides cinchona, el desarrollo de nuevas CSPs que permitan obtener tiempos de análisis reducidos y alta eficacia, y trabajar en distintos modos de separación, presenta un gran interés. Las columnas monolíticas han atraído un interés creciente en los últimos años debido al amplio intervalo de pH en el que pueden trabajar, las bajas presiones que se obtienen a altos flujos, la alta eficacia y sensibilidad que proporcionan, el bajo consumo de disolventes y su fácil acoplamiento a Espectrometría de Masas.

Las columnas monolíticas quirales pueden dividirse en tres categorías de acuerdo a los materiales en los que se basan: columnas monolíticas basadas en sílice, columnas monolíticas basadas en polímeros y columnas monolíticas híbridas orgánico-sílices. En comparación con las columnas monolíticas basadas en polímeros o las híbridas, las columnas monolíticas basadas en sílice presentan limitaciones debido a su complicado y laborioso proceso de preparación, por estar afectadas por el envejecimiento o el tratamiento con calor, y por su fácil contracción o hinchamiento en disolventes orgánicos, mientras las primeras han constituido un tema de investigación muy relevante.

Por otra parte, aunque existen muchas posibilidades para llevar a cabo con éxito una separación quiral, las técnicas de micro-separación juegan un papel vital debido a las ventajas derivadas de sus reducidas dimensiones. Entre las distintas estrategias miniaturizadas para llevar a cabo separaciones quirales, el empleo de columnas quirales en nano-cromatografía de líquidos (nano-LC) o electrocromatografía capilar (CEC) ha atraído mucho interés. Nano-LC es un desarrollo cromatográfico relativamente nuevo impulsado por los recientes avances producidos en aplicaciones biológicas y en investigación proteómica que requieren utilizar reducidos diámetros internos en columnas de cromatografía de líquidos (LC) y que permiten el análisis de pequeñas cantidades de muestra, así como incrementar la sensibilidad. En comparación con HPLC convencional, nano-LC no solo puede disminuir considerablemente el consumo de disolventes, muestras y residuos, sino que puede hacer también posible obtener mayores eficacias de separación, buena resolución y

tiempos de análisis más cortos. Hasta ahora, nano-LC se ha empleado con columnas monolíticas para la separación de proteínas, enantiómeros y otras sustancias. El objetivo de este trabajo de investigación ha sido el desarrollo de columnas monolíticas quirales, su evaluación y su aplicación en nano-LC. Para ello, se han seleccionado tres selectores quirales como la vancomicina, teicoplanina y O-[2-(metacriloiloxi)-etilcarbamoil]-10,11-dihidroquinidina (MQD) para la preparación de las columnas monolíticas.

Los antibióticos macrocíclicos del tipo de la vancomicina han demostrado ser selectores quirales versátiles para llevar a cabo separaciones enantioméricas. Muchos artículos han descrito el empleo de vancomicina para separaciones quirales. Sin embargo, la preparación de columnas monolíticas basadas en polímeros funcionalizados con vancomicina apenas se ha descrito. Hasta el momento, solo se ha desarrollado una estrategia de modificación post-columna multietapa para inmovilizar vancomicina en la superficie de una columna monolítica polimérica. Aunque las columnas monolíticas basadas en polímeros funcionalizados con vancomicina preparadas siguiendo este procedimiento demostraron tener una buena enantioselectividad para compuestos racémicos por CEC, se pusieron de manifiesto algunos inconvenientes asociados como su lentitud, laboriosidad y baja repetibilidad.

Con el fin de reducir el tiempo asociado a la fabricación de columnas monolíticas basadas en polímeros orgánicos funcionalizados con vancomicina y reducir la complejidad de su preparación, en esta Tesis Doctoral se ha desarrollado una aproximación de co-polimerización basada en un solo paso. Con este fin, el derivado 2-isocianatoetil metacrilato (ICNEML) de vancomicina (ICNEML-vancomicina) se sintetizó y a continuación se co-polimerizó con el agente entrecruzante y el iniciador en un sistema porogénico binario para preparar la columna monolítica polimérica funcionalizada con vancomicina y denominada poli(ICNEML-vancomicina-*co*-EDMA). Las condiciones de polimerización se optimizaron sistemáticamente para obtener permeabilidad, eficacia de columna y resolución enantiomérica satisfactorias. La capacidad de enantioresolución de la columna monolítica optimizada se evaluó analizando una serie de fármacos quirales en los modos de fase polar orgánica y fase reversa. Asimismo, se seleccionaron las condiciones más adecuadas para llevar a cabo las separaciones enantioméricas incluyendo el tipo y concentración del disolvente orgánico, la concentración de la disolución reguladora y pH de la fase móvil, siguiendo un cuidadoso proceso de optimización.

Teicoplanina es otro importante selector quiral del grupo de los antibióticos macrocíclicos que posee una superficie más activa que otros glicopéptidos relacionados debido a la cadena hidrocarbonada N-acil en la glucosamina. Sin embargo, la mayor parte de los trabajos descritos se han centrado en columnas empaquetadas con sílice modificada con teicoplanina. Solo dos agliconas de teicoplanina (TAG) se han preparado en una columna monolítica mediante la aproximación partícula modificada. Aunque estas columnas demostraron ser útiles para llevar a cabo la separación de glicil-dipéptidos, aminoácidos y dipéptidos diestereoméricos, la limitación de esta aproximación es que la dispersión de las partículas en la mezcla de polimerización es baja dado que los materiales tienen tendencia a depositarse cuando se usan en porcentajes altos. Además, en nuestro grupo de investigación se preparó una columna monolítica poli(ICNEML-teicoplanina-*co*-EDMA) por el método de polimerización *in situ* en un solo paso similar al utilizado en la preparación de la columna poli(ICNEML-vancomicina-*co*-EDMA). Sin embargo, la columna resultó no ser estable en el modo de fase polar orgánica. Por lo tanto, el desarrollo de columnas monolíticas funcionalizadas con teicoplanina que superen estos problemas es de gran interés.

En esta Tesis Doctoral se ha preparado una columna monolítica híbrida orgánico-sílice funcionalizada con teicoplanina mezclando el monómero de copolimerización teicoplanina-2-isocianatoetil metacrilato (Tei-ICNEML) y el iniciador en la disolución de hidrólisis de tetrametil ortosilicato (TMOS) y 3-(trimetoxisilil)-propilmetacrilato (γ-MAPS). Las condiciones de preparación y separación se optimizaron de forma sistemática y la columna monolítica óptima se aplicó a la separación enantiomérica de compuestos quirales en los modos de fase polar orgánica (POM) y de fase inversa (RPM).

Recientemente, los derivados de alcaloides cinchona se han empleado como selectores quirales como es el caso de las columnas monolíticas basadas en quinina y quinidina desarrolladas por el grupo de Lämmerhofer y re-optimizadas sistemáticamente por el grupo de Jiang. Las columnas monolíticas así preparadas demostraron tener una buena enantioselectividad para la mayor parte de los aminoácidos N-derivatizados estudiados, péptidos de pequeño tamaño N-derivatizados y herbicidas. Sin embargo, los trabajos realizados se centraron en dos tipos de columnas basadas en sílice y en polímeros orgánicos y solo algunas de ellas se basaban en aproximaciones multi-etapa para preparar columnas monolíticas

híbridas orgánico-sílice funcionalizadas con quinina. Además, el objetivo de estos trabajos fue principalmente describir la estrategia de preparación de estas columnas monolíticas y su capacidad para separar enantioméricamente varios aminoácidos N-derivatizados siendo muy escasa su aplicación al análisis de muestras reales.

Con el fin de conseguir preparar una columna monolítica híbrida orgánico-sílice funcionalizada con quinidina y simplificar los pasos previamente utilizados para la preparación de columnas monolíticas híbridas basadas en quinina, en esta Tesis Doctoral se seleccionaron derivados de quinidina (MQD) como selectores quirales para la preparación de columnas monolíticas híbridas orgánico-sílice mediante una estrategia fácil de un solo paso. Se evaluó el efecto de la composición de la fase móvil en la columna monolítica híbrida MQD-sílice optimizada en los modos de fase polar orgánica y fase reversa y se llevó a cabo la separación enantiomérica de un total de 52 aminoácidos proteicos y no proteicos N-derivatizados en los dos modos de separación.

Los aminoácidos son compuestos esenciales que juegan un papel vital en muchos organismos vivos y tanto para aminoácidos proteicos como no proteicos se han descrito diferencias en las propiedades de los D- y los L-enantiómeros. Aunque existen columnas cromatográficas, incluso una amplia variedad de columnas comerciales, que permiten llevar a cabo la separación enantiomérica de aminoácidos, la aplicabilidad de columnas monolíticas híbridas basadas en quinidina al análisis de aminoácidos en muestras reales no se había descrito. Por ello, en esta Tesis Doctoral, la columna monolítica híbrida MQD-sílice desarrollada se ha aplicado a la separación de aminoácidos proteicos y no proteicos por nano-LC. Con el fin de obtener una capacidad de enantioresolución satisfactoria, se han investigado distintos agentes derivatizantes y se han optimizado las condiciones cromatográficas. Finalmente, se analizaron 27 aminoácidos, 19 proteicos y 8 no proteicos utilizando FMOC como agente derivatizante, se han evaluado las características analíticas del método desarrollado y se ha aplicado a la determinación de dos L-aminoácidos en suplementos dietéticos.

SUMMARY

Chiral separations have been a hot topic for a long time and continue being a field of high interest nowadays due to their high impact in different fields such as the pharmaceutical, agrochemical and food industry, among others. Numerous chiral stationary phases (CSPs) are commercially available, including those based on cyclodextrins, polysaccharides, 3,5-dinitrobenzamide-naphthylglycine derivatives, proteins, macrocyclic antibiotics or cinchona alkaloids. However, in order to achieve short analysis times, high efficiency, and multi-separation modes, the development of novel CSPs presents a high interest.

Monolithic columns have been attracted more and more attention in the last years due to their wide range of pH suitability, low backpressure in the high flow rate, high separation efficiency and sensitivity, low sample and elution solvent consumption, as well as the ease coupling to Mass Spectrometry. According to the different matrix materials, these chiral monoliths can be divided into three categories, such as silica-based monolithic columns, polymer-based monolithic columns and organic-silica hybrid monolithic columns. Compared with the polymer-based or organic-silica hybrid monolithic columns, the silica-based monolithic columns are limited because of their complicated and time-consuming preparation process, for being affected during the aging and heat-treatment, and easily shrinking or swelling in organic solvents, while the polymer-based or organic-silica hybrid monolithic columns have become research hotspot.

Many possibilities are available to carry out a successful chiral separation, in which micro-separative techniques play a vital role due to the advantages derived from their inherent low dimensions. Among the different miniaturized strategies to achieve chiral separations, the use of chiral columns in nano-liquid chromatography (nano-LC) or capillary electrochromatography (CEC) has attracted much interest. Nano-LC is a relatively new development in chromatography driven by the recent advances in biological applications and proteomics research that required decreasing inner diameters (I.D.) of liquid chromatography (LC) columns to allow for a smaller sample amount and to increase sensitivity. Compared with conventional HPLC, nano-LC not only can significantly decrease the consumption of solvents, samples, and production of waste, but also make possible higher separation efficiency, good resolution and shorter analysis times. So far, nano-LC combined with monolithic columns has been widely used in the separation of proteins, small structure compounds enantiomers and others substances. This research work is aimed to the development of chiral monolithic

columns and their evaluation and application in nano-LC. Hence, three chiral selectors including vancomycin, teicoplanin, and O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine (MQD) were selected for the preparation of the monolithic columns.

Vancomycin-type macrocyclic antibiotics have shown to be versatile chiral selectors to achieve enantiomeric separations. Over the years, many articles reported that vancomycin was used for chiral separation. However, the preparation of vancomycin functionalized polymer-based monoliths has scarcely been described. So far, only a multi-step post-column modification strategy was developed for immobilizing vancomycin onto the surface of an organic polymeric monolith. Although vancomycin functionalized polymer-based monoliths prepared following this procedure exhibited good enantioselectivity for racemic compounds in CEC, some disadvantages associated with this strategy were pointed out, such as be time-consuming, laborious and be related with unsatisfactory repeatability.

In order to reduce the time associated with the fabrication of vancomycin functionalized organic polymer-based monoliths and to reduce the complexity of their preparation, a single-step co-polymerization approach was developed in this PhD Thesis. With this aim, the 2-isocyanatoethyl methacrylate (ICNEML) derivative of vancomycin (ICNEML-vancomycin) was first synthesized, and then co-polymerized with the cross-linker and initiator in a binary porogen system to prepare the polymer-based vancomycin functionalized monolithic column namely poly(ICNEML-vancomycin-*co*-EDMA). Polymerization conditions were systematically optimized to obtain satisfactory permeability, column efficiency and enantiomeric resolution. The enantioresolution capability of the optimized monolith was evaluated by analyzing a series of chiral drugs in polar organic-phase and reversed-phase modes. The most adequate conditions to achieve the enantiomeric separations, including the type and concentration of organic solvent, the buffer concentration and the pH of the mobile phase, were selected through a carefull optimization procedure.

Teicoplanin is another important macrocyclic antibiotic chiral selector, and it has more active surface than other related glycopeptides due to the N-acyl hydrocarbon chain on the one glucosamine. However, most of the reports were concentrated on teicoplanin modified silica packed columns. Only two teicoplanin aglycone (TAG) were prepared into the monolith by the particle-loaded approach. Although they exhibited a good enantioseparations for several glycyl-dipeptides, amino acids and

diastereomeric dipeptides, the limitation of this particle loaded approach is that the dispersion of particles in the polymerization mixture is poor since materials are prone to settling when used in higher percentages. In addition, a poly(ICNEML-teicoplanin-*co*-EDMA) monolithic column was also prepared by our research team *via* single-step with *in-situ* polymerization, which is similar with the preparation of poly((ICNEML-vancomycin-*co*-EDMA). However, the column was unstable in polar organic phase mode. Therefore, the development of novel teicoplanin functionalized monolithic columns to overcome these problems is very meaningful.

In this PhD Thesis, a teicoplanin organic-silica hybrid monolithic column was prepared by mixing the copolymerization monomer teicoplanin-2-isocyanatoethyl methacrylate (Tei-ICNEML) and initiator into the hydrolysis solution of tetramethyl orthosilicate (TMOS) and 3-(trimethoxysilyl)-propylmethacrylate (γ-MAPS). The preparation and separation conditions were systematically optimized. The optimum monolithic column was applied to achieve the enantioseparation of chiral compounds under the polar organic-phase (POM) and reversed-phase (RPM) modes.

Recently, a series of cinchona alkaloid derivatives were used as the chiral selector, such as quinine and quinidine-based monolithic columns developed by Lämmerhofer's group, and systematically re-optimized by Jiang's group. The resulting monoliths exhibited good enantioselectivity for most of the studied *N*-derivatized amino acids, *N*-derivatized small peptides, and herbicides. However, all the reports mainly focused on two types which were silica-based and organic polymer-based, while only a few have been reported so far being based on so called multistep approaches to preparation of the quinine functionalized organic-silica hybrid monolithic column. Moreover, the aim of those works was mainly to describe the preparation strategy for those monolithic columns and their ability to enantioseparate several *N*-derivatized amino acid racemic standards, while their application in the real samples is rarely reported.

In order to fill the gap which is lack of the quinidine functionalized organic-silica hybrid monolithic column, and simplify the steps which was previously used for the preparation of quinine hybrid monolithic columns, we selected the quinidine derivatives (MQD) as chiral selector to preparation of an organic-silica hybrid monolithic column through a facile "one-step" strategy. Finally, the composition of mobile phase was evaluated on the optimized MQD-silica hybrid monolithic column under the RPM and POM conditions, and a total of 52 N-derivatized protein and non-protein amino acids were enantioseparated under both mobile phase modes.

Amino acids are essential and ubiquitous compounds playing a vital role in many living organisms, and the differences in the properties of D- and L-enantiomers have been widely reported, both for protein and non-protein amino acids. Although there are many chromatography columns for the enantiomeric analysis and separation of amino acids, which even include many commercial chromatography columns, the applicability of quinidine silica-hybrid monolithic columns to the quantitative analysis of amino acids in real samples has not been demonstrated yet. Therefore, the MQD-silica hybrid monolithic column was applied to the enantiomeric separation of protein and non-protein amino acids by nano-LC. In order to obtain satisfactory enantioresolution performance, we examined different reagents for amino acid derivatization, and optimized the chromatographic conditions. Finally, 27 amino acids were tested consisting of 19 protein and 8 non-protein amino acids using FMOC as derivatizing reagent, the analytical characteristics of the developed method were evaluated, and the method was applied to the enantiomeric determination of two different amino acids in dietary supplements.

ACRONYMS, ABBREVIATIONS AND SYMBOLS

AA	Allyl amine
ACN	Acetonitrile
ADDAB	Allyldimethyldodecylammonium bromide
AGE	Allyl glycidyl ether
AGP	α-acid glycoprotein
AIBN	Azobisisobutyronitrile
Ala	Alanine
Arg	Arginine
Asp	Aspartic acid
AVI	Avidin
B	Benzoyl
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CDI	Carbonyldiimidazole
CDMPC	Cellulose tris(3,5-dimethylphenyl carbamate)
CEC	Capillary electrochromatography
CEC	Capillary electrochromatography
CHCl3	Chloroform
CLEC	Chiral ligand-exchange type chromatography
CLIP	Clindamycin phosphate
CSPs	Chiral stationary phases
c-SWNTs	Carboxylated single-walled carbon nanotubes
CTAB	Cetyltrimethylammonium bromide
CTMB	Cellulose tris(4-methylbenzoate)
DATDA	N,N'-diallyl-L-tartardiamide
DMSO	Dimethyl sulfoxide
DNB	Dinitrobenzoyl
DNS	Dansyl
DS	Degree of substitution
DSC	Disuccinimidyl carbonate

EDA-β-CD	Ethylenediamine-β-CD
EDMA	Ethylene dimethacrylate
EG	Ethylene glycol
EP	(±)-ephedrine
FMOC	9-fluorenylmethoxycarbonyl
GMA	Glycidyl methacrylate
GO	Graphene oxide
H_3BO_3	Boric acid
HAc	Acetic acid
HEMA	2-hydroxyethyl methacrylate
HSA	Human serum albumin
ICNEML	2-isocyanatoethyl methacrylate
ICNPTES	3-isocyanatopropyltriethoxysilane
Isoleu	Isoleucine
LC	Liquid chromatography
LE	Ligand-exchange
Leu	Leucine
LOD	Limit of detection
LOQ	Limit of quantification
L-PheA	L-phenylalaninamide
L-ProA	L-prolinamide
MBQD	*O*-9-(*tert*-butylcarbamoyl)-11-[2-(methacryloyloxy)ethylthio]-10,11-dihydro-quinidine
MeOH	Methanol
Met	Methionine
MI	Molecular imprinting
MQD	O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine
N	Theoretical plate number
NBD	7-nitro-2,1,3-benzoxadiazole
NH_2-MSNs	Amino-modified mesoporous silica nanoparticles
NH3·H2O	Ammonium hydroxide

NSAIDs	Nonsteroidal anti-inflammatory drugs
OVM	Ovomucoid
PEG	Polyethylene glycol
PEP	(±)-Pseudoephedrine
PGA	Penicillin G acylase
PMA	Propargyl methacrylate
PMPMS	Poly-3-mercaptopropyl methylsiloxane
poly(NAS)	N-acryloxysuccinimide
POM	Polar organic phase mode
POSS-PEI	Polyhedral oligomeric silsesquioxane-poly(ethylenimine)
Pro	Proline
RPM	Reversed phase mode
Rs	Enantioresolution
SAADCL	Sodium 10-acryl-amidodecenoxy carbonyl-L-leucinate
SAADoCL	Sodium 12-acryl-amidododecenoxy carbonyl-L-leucinate
SAAOCL	Sodium 8-acrylamidooctenoxy carbonyl-L-leucinate
SEM	Scanning electron microscopy
Ser	Serine
TEA	Triethylamine
THF	Tetrahydrofuran
TMOS	Tetramethoxysilane
TM-β-CD	Trimethylated-β-CD
TVCH	1,2,4-trivinylcyclohexane
Val	valine
VTMS	Vinyltrimethoxysilane
ZHM	Zirconia hybrid monolith
ZM	Zirconia-based monolith
α	Enantioselectivity
γ-MAPS	3-(trimethoxysilyl)-propylmethacrylate
k	Retention factor

K	Permeability
m/z	Mass to charge ratio
m-CIB	*m*-chlorobenzoyl
p-CIB	*p*-chlorobenzoyl
p-NB	*p*-nitrobenzoyl
t-BuCQD	*O*-9-(*tert*-butylcarbamoyl)quinidine
t-BuCQN	*O*-9-tert-butylcarbamoylquinine
3,5-DCIB	3,5-dichlorobenzoyl
3,5-DMB	3,5-dimethoxybenzoyl
3,5-DNB	3,5-dinitrobenzoyl
3-GPTM	3-glycidoxypropyltrimethoxysilane

TABLE OF CONTENTS

CHAPTER I
INTRODUCTION

I.1. Monolithic columns

A monolithic column is a rigid macroporous monolith prepared in a capillary column of 20-400 μm inner diameter by an *in situ* polymerization [1]. Regarding the origin of the monolithic materials for separation, the first record can be traced back to early 1950s [2]. In 1992, the first monolithic column was prepared into the tube of a chromatographic column by Svec and Fréchet, so from this date the development of monolithic columns entered a golden age [1]. According to the needs of the separation, a monolithic column can be functionalized with different monomers and prepared to form different pore sizes. These novel chromatographic separation materials have attracted more and more attention due to the simple strategy necessary for their preparation, and their excellent permeability, pH stability, low resistance to mass transfer, and high performance [3]. For these reasons, they have widely been used for the separation of a great variety of compounds, including small structure compounds (such as benzoic acids derivatives [4], vitamins [5], nonsteroidal anti-inflammatory drugs (NSAIDs) [6]), enantiomers (β-blockers [7], amino acids [8], mandelic acid derivatives [9]), phenols [10] or peptides [11].

Recently, the focus of attention of the research in this field has been to adopt different preparation strategies and to introduce different matrix materials into the capillary to control the chemical properties of the monolithic surface. For this reason, a large number of monolithic columns has been developed for chromatographic separations, and some of them have been commercialized, such as the CHIRALPAK® QD-AX, Silica ROD™, Prep RODs™, Chromolith™, CHIRALPAK®ZWIX (-) and CIM™. According to the different matrix materials, monolithic columns can be divided into three categories:

(a) silica-based monolithic columns [12, 13], prepared by the sol-gel approach, including hydrolysis, condensation, ageing, and baking. These monolithic columns exhibited a wide range of pH suitability, low backpressure at high flow rate, and significantly shorter run times. However, the cumbersome and multi-step preparation method makes the preparation cycle longer. In addition, in order to modify the different monomers on the monolithic column surface, a troublesome step was necessary to treat the bed.

(b) polymer-based monolithic columns [1, 14], prepared by mixing the monomers, porogens, cross-linker and initiator. This approach is not only simpler, but also has the advantage that the monomers that can be selected are more extensive than for the silica-

based monolithic columns. However, these monolithic columns are prone to swell in the organic solvent, present low mechanical strength, and poor stability due to the nature of the material.

(c) organic hybrid-based monolithic columns [15-17], usually prepared by the sol-gel approach using two different silanization reagents, one is the trialkoxysilane which is functionalized, and the other is the tetraalkoxysilane used as the cross-linker. These monolithic columns overcome the problem of swelling in the organic solvent and, in addition, due to the introduction of the functionalized trialkoxysilanes, their preparation does not require post-derivatizaton to modify the monomers in the monoliths bed.

As an important factor for successful enantioresolution, the development of chiral stationary phases (CSPs) runs through the history of chiral separation. Although there are a wide range of commercially available CSPs, great efforts are still directed towards developing novel CSPs to meet the requirements of fast analysis, dimensions minimization and usefulness in different separation modes. Over the last two decades, increasing attention has been paid to monolithic CSPs, due to their inherent advantages over traditional particle-based columns, such as high permeability, low resistance to mass transfer, and simple preparation within micro- or nano-formats. Chiral selectors were normally immobilized onto the surface of the monolithic matrix through covalent bonding [18], physical coating [19, 20], or particle fixing [21, 22], among others. So far, organic polymer-based, silica-based, and organic-silica hybrid monolithic CSPs have all been reported. However, most of them were focused on the preparation of capillary chiral monolithic columns. Although several important review articles well summarized the development and classification of monolithic CSPs according to the type of matrix employed, a thorough overview of their preparation methods is still absent.

By surveying the relevant keywords “chiral stationary phase” and “monolithic column” in the SciFinder and the Web of Science, there are more than one hundred articles related to this topic. According to the content of all these articles, they can be divided into two directions, one is the preparation of novel monolithic columns, another is the applications for the separation. Although numerous monolithic columns are commercially available, the development of novel monolithic columns presents a high interest. In the next sections, the recent developments related to monolithic columns will be described with particular emphasis on the classification and preparation of chiral monolithic columns.

I.2. Classification of monolithic columns

I.2.1. Silica-based monolithic columns

The preparation of silica-based monoliths was reported as early as 1990s [23]. In 1991, Nakanishi *et al*. [24] first prepared silica-based monoliths with different morphological structures using poly (sodium styrenesulfonate) aqueous solutions, nitric acid and tetramethoxysilane. Although their subsequent work was limited to discuss the preparation conditions and pore size control, they opened a new research direction for the development of monolithic materials applied to chromatographic separations.

In 1996, Minakuchi *et al.* [25] first prepared silica rods for HPLC and this marked the moment that silica-based monolithic columns entered officially into the chromatographic field. Subsequently, a series of silica monolithic columns were developed for HPLC, CLC and CEC by Fields *et al.* [26], Ishizuka *et al.* [27], Dulay *et al.* [28]. So far, there have been a large number of articles reporting the preparation and applications of silica monolithic columns, even some of them have been commercialized, such as Silica ROD™, Prep ROD™, and Chromolith™, among others.

Regarding silica-based monolithic columns, the most widely used preparation approach was the sol-gel method, which can be summarized into five steps [12] as shown in **Fig. 1**: (a) preparation of the sol solution, TMOS and TEOS were usually selected as the precursor, PEG was used as the porogen, and they were mixed into an acidic aqueous solution; (b) sol solvent phase separation and gelation, (c) aging and solvent exchange, (d) evaporation drying, and (e) heat-treatment under high temperature.

In order to get the different separation function for the silica-based monolith, usually, it is necessary to modify it so that the functional monomer can be introduced into the monolithic column. Hence, a variety of silane reagents for silica-based monolithic stationary phases has been used [29], as shown in **Fig. 2**.

Although these columns had a wide range of pH suitability, low backpressure, high porosity, and good thermal stability, they also had some disadvantages: (a) first, the preparation process was very complicated and time-consuming; (b) the morphology of silica-bed was easily affected during the aging and heat-treatment; (c) the skeleton was easily shrinking or swelling in organic solvents [13, 30].

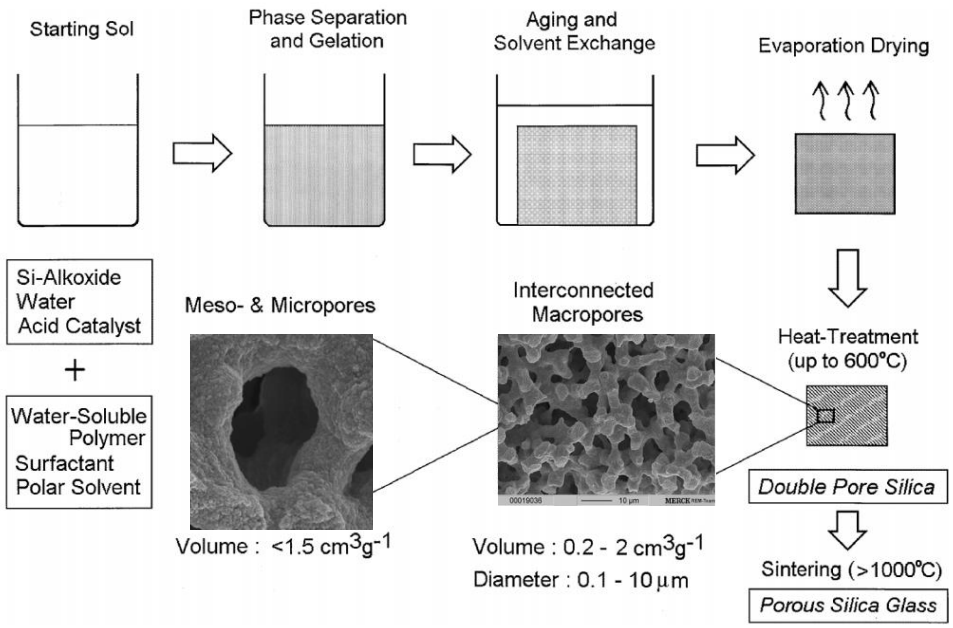


Fig. 1. Schematic flow chart of the silica-based monolith preparation procedure [12].

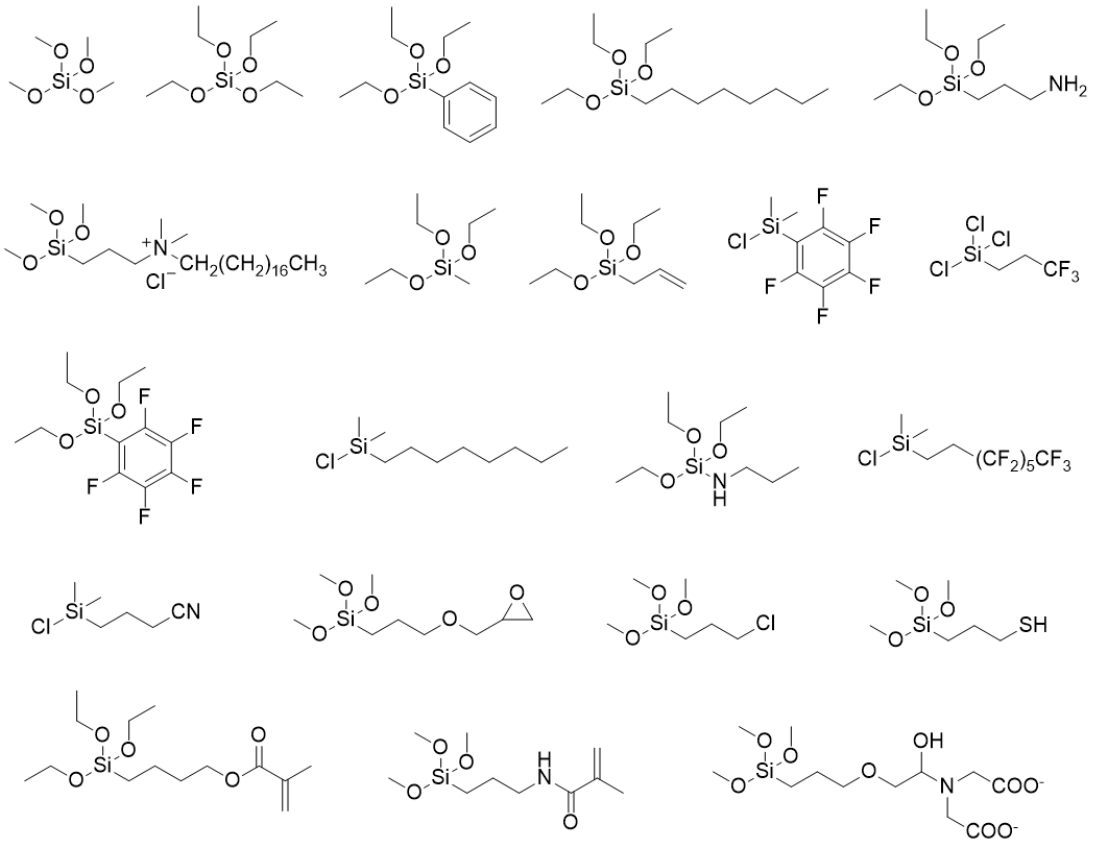


Fig. 2. List of silane reagents used to prepare silica-based monolithic stationary phases [29].

I.2.2. Polymer-based monolithic columns

Simplifying the preparation of monolithic columns has always been a very interesting thing, and polymer monolithic columns meet such requirements. Since the 1950s, the concept of polymer monolith which can be used in chromatography has been proposed by Synge *et al.* [2]. However, the first polymer monolithic column was prepared by Sves *et al.* in the 1990s [1]. The continuous porous polymer rod was prepared using the monomer (glycidyl methacrylate and ethylene dimethacrylate), porogens (cyclohexanol and dodecanol) and initiator (azobisisobutyronitrile) *via in situ* polymerization.

Based on these previous works, a lot of polymer-based monolithic columns were developed with different functional monomers [14, 31, 32]. As **Fig. 3** shows, this simple method just needs (a) mixing the functional monomer, cross-linker, porogens and initiator, (b) after degassing, introducing the pre-polymerization mixture into the capillary for *in situ* polymerization under illumination or heating, (c) rinsing out of the unreacted monomer and porogens to obtain the porous organic polymer monolithic column.

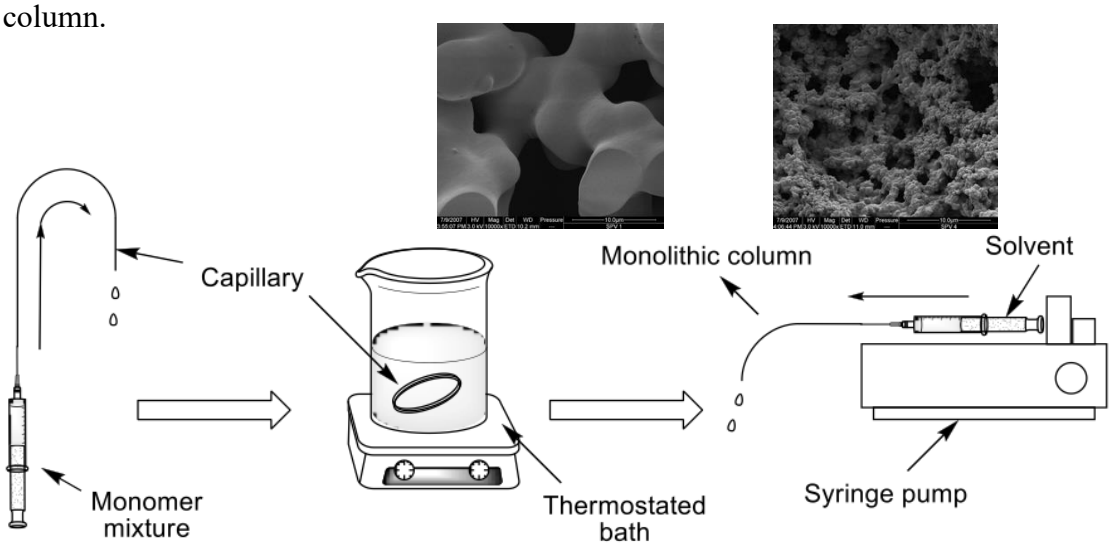


Fig. 3. Schematic flow chart of the polymer-based monolith preparation procedure.

Polymer-based monolithic columns can be divided into three categories depending on the functional monomer employed in the preparation: acrylamide-based, styrene-based, and methacrylate-based monoliths [14].

In the case of acrylamide-based monolithic columns, acrylamide, butyl methacrylate, *N*-isopropylacrylamide, and methacrylamide can be selected as monomers, *N,N′*-methylenebisacrylamide and piperazine diacrylamide are usually employed as the cross-linker. Although these polymer-based monolithic columns exhibited good permeability and efficiency, the mechanical stability was unsatisfactory. Additionally,

due to the use of an aqueous phase as the polymerization system, many hydrophobic monomers cannot be dissolved in the solvent, which also limits their development.

Styrene-based monolithic columns were also prepared by free radical copolymerization of monomers and cross-linker. However, compared with other polymer-based monolithic columns, the monomers that can be used were very limited, just including styrene divinyl benzene and vinylbenzylchloride. More importantly, this styrene-based monolithic columns were difficult to modify on their surface due to the lack of reactive groups.

Generally, methacrylate-based monolithic columns were prepared using an alkyl methacrylate functional monomer, such as butyl methacrylate, glycidyl methacrylate (GMA), 2-hydroxyethyl methacrylate and so on, which was co-polymerized with cross-linker when the AIBN was used as the initiator. The (ethylene dimethacrylate (EDMA)) was usually selected as the cross-linker, and the porogen was available in a variety of options, such as MeOH, 1-propanol, tetrahydrofuran (THF), 1,4-butanediol, DMSO, ACN, and it also can be freely combined according to functional monomer. Due to the simple preparation, diversity of monomers, and good mechanical stability, these monolithic columns have been the most widely used.

I.2.3. Organic-silica hybrid monolithic columns

Although the above-mentioned two types of monolithic columns have been widely used for chromatographic separation, their main drawback is that they are prone to swelling in the organic solvents [33, 34]. In order to solve this problem, a novel monolithic column namely organic-silica hybrid monolithic column was developed by Hayes *et al.* [15]. This monolith column combines the advantages of both silica-based and organic polymer-based monolithic columns, including the high mechanical stability, and easily preparation and modification. After that, Zou's group reported a lot of works dealing with these new hybrid columns [16, 17].

Briefly, the organic silica hybrid monolithic column was prepared by two processes (shown in **Fig. 4**): (a) methoxy groups from tetraalkoxysilane (such as TMOS and TEOS) and functional trialkoxysilane reagents are hydrolyzed into silanols under acidic or basic conditions, then (b) the silanol groups from the silanization reagents and inner wall of the capillary are polycondensated under the suitable temperature.

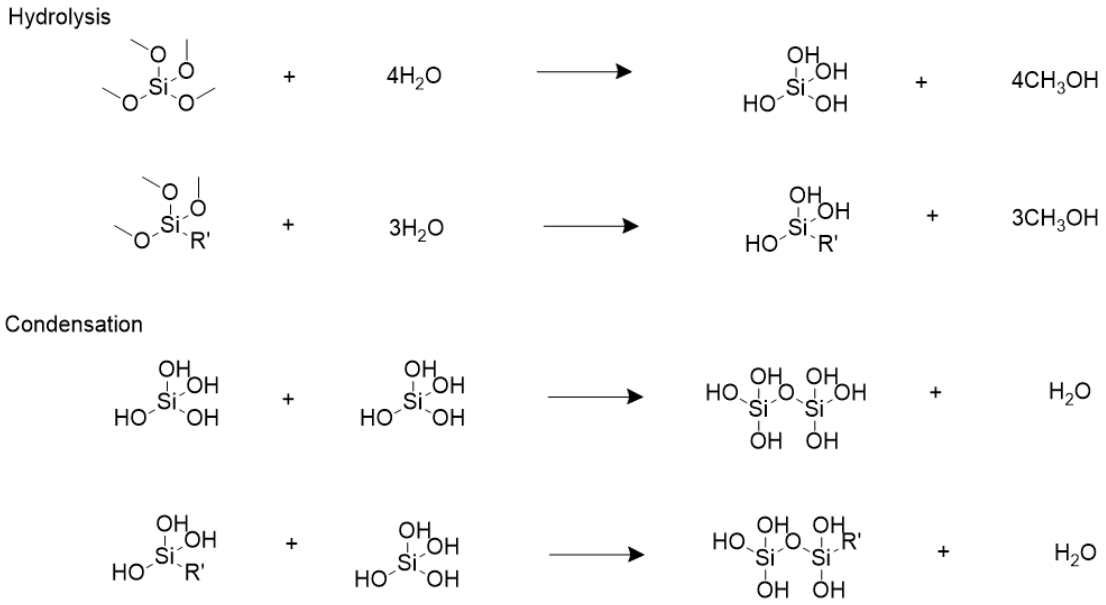


Fig. 4. Schematic flow chart of the sol-gel procedure.

So far, there is a large number of functional trialkoxysilane reagents to be selected [35] as shown in **Fig. 5**. Due to the introduction of some trimethoxysilylation reagents which are boned special groups, such as epoxy group, mercapto group, amino group, isocyano group, among others, Zou *et al.* [16, 17] developed the "one-pot" approach to prepare the hybrid monolithic column. They just needed to mix TMOS and VTMS (or MAPS, GPTMS, etc.) into the acidic aqueous solution containing PEG. After the end of the hydrolysis, the functional monomers and AIBN are added into the hydrolysis solution, and the condensation and polymerization process can be controlled by adjusting the temperature.

I.3. Chiral monolithic columns

After about 20 years' efforts, the monolithic counterparts of most traditional particulate CSPs, as well as some unique monolithic CSPs, were described in the literature. Based on the type of chiral selectors, the monolithic CSPs can be mainly classified into the following categories: cyclodextrin-functionalized, polysaccharide-functionalized, protein-functionalized, antibiotic-functionalized, chiral ligand-exchange, chiral ion-exchange, brush-type as well as molecularly imprinted monolithic CSPs. In this section, the applications of these different types of chiral selectors are summarized in silica-based, polymer-based and organic-silica hybrid-based monolithic columns.

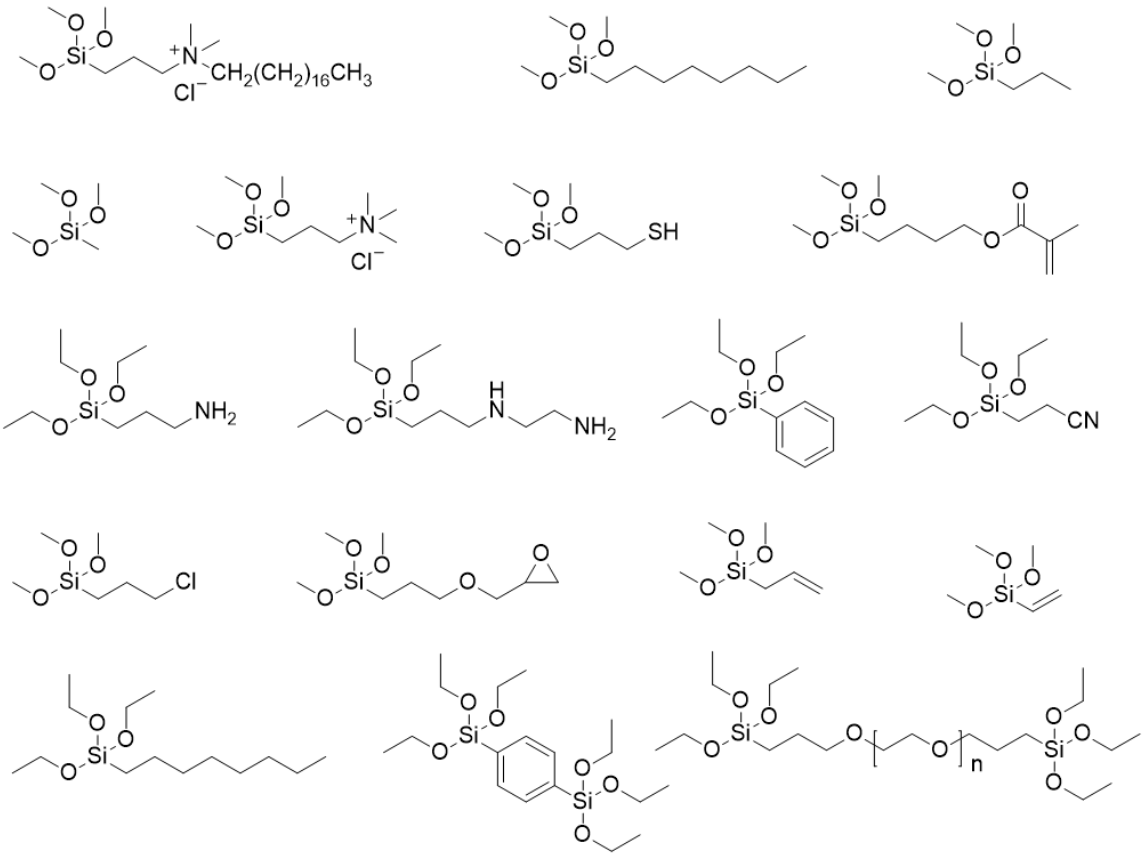


Fig. 5. List of silane reagents for silica-based monolithic stationary phases [35].

I.3.1. Cyclodextrins and cyclodextrin derivatives

Cyclodextrins (CDs) and their derivatives are widely used as chiral selectors in CE and GC, but are relatively less popular in HPLC. Surprisingly, CD-functionalized monoliths are probably the most frequently studied monolithic CSPs so far (**Table 1**). Both organic polymer-based and silica-based CD-functionalized monolithic CSPs have been developed [36, 37]. Zirconia-based [7, 38] and organic-silica hybrid [8] monoliths have also been recently introduced.

I.3.1.1. Polymer-based CD monolithic columns

Koide and Ueno [39] have pioneering contributions to the preparation of monolithic CSPs with CDs as selectors. These authors developed several chiral charged polyacrylamide gels by incorporating CDs or their derivatives into homogeneous gels during a single-step copolymerization. Although these chiral homogeneous gels exhibited a certain enantioselectivity in capillary electro-chromatography (CEC) mode, their low permeability restricted their further applications in capillary liquid chromatography (CLC) mode [9]. Therefore, the CD-functionalized rigid monoliths,

particularly acrylate or methacrylate based, have become mainstream nowadays because of their good permeability and stability. In the studies dealing with the preparation strategy, native CD-functionalized organic polymer-based monoliths were more commonly investigated [9, 40-44]. However, their enantioselectivity was unsatisfactory.

In order to overcome this problem, a series of monolithic CSPs based on different CD derivatives, including aromatic β-CD [45, 46], carboxymethyl-β-CD (CM-β-CD) [19], methylated β-CD [47, 48], carbamoylated β-CD [46, 49-51], acetyl-β-CD [20], aspartate-β-CD (Asp-β-CD) [9], hydroxypropyl-β-CD (HP-β-CD) [9] (**Fig. 7**), were also developed. Fu *et al*. [9] reported a series of CD-functionalized monoliths, based on β-CD and its derivatives (Asp-β-CD and HP-β-CD), for the enantioseparation of eight amino acids and two chiral drugs. Both the HP-β-CD and Asp-β-CD functionalized monolithic columns exhibited better chiral recognition ability than the β-CD based monolith (their enantioresolution for tyrosine was 4.11, 2.45 and 1.53, respectively).
On the other hand, Li and coworkers [52] employed gold nanoparticles (GNPs) with high surface area to prepare β-CD-functionalized organic polymer-based monolithic CSPs aimed at obtaining higher density of the immobilized β-CD and then higher enantioselectivity. β-CD-modified gold nanoparticles (BCD-GNPs) were covalently bonded onto the thiolated surface of the porous polymer. It was found that the surface area of the resulting polymer (24.12 m^2/g) increased in comparison with the monolithic column without addition of BCD-GNPs (15.17 m^2/g). For zopiclone, the theoretical plate number and enantioresolution were 128,000 plates/m and 1.85. However, the expected enhancement in enantioseparation performance of the BCD-GNP-functionalized monolith over the conventional β-CD-functionalized monolith was not observed.

I.3.1.2. Silica-based CD monolithic columns

Wistuba and Schurig developed the particle-fixation technique which was the basis for the preparation of silica-based CD monolithic column [21]. Although the chiral selector (Chirasil-Dex) was incorporated into the silica bed by sintering approach, the resulting column exhibited good long term stability over four months continued use in terms of resolution and retention. On the basis of this method, they used the sol-gel approach to prepare a permethylated β-CD functionalized monolithic column by glutting Chirasil-Dex grafted silica particles together inside the capillary [22]. Column

performance was evaluated using chiral analytes such as barbituric acids, herbicides and nonsteroidal NSAIDs in both CEC and CLC modes. The highest enantioselectivity (α) and enantioresolution (Rs) values were 1.15 and 2.68 for mephobarbital, respectively. However, the intrinsic disadvantages of these columns, such as low permeability, high back pressure and tedious packing procedure, restricted their further popularization and application. Later on, a sol-gel based post-modification was employed for the fabrication of CD-functionalized silica-based monolithic CSPs, such as native β- and γ-CD [53], phenyl carbamate β-CD [6] and sulfated β-CD [18], owing to their higher permeability, lower pressure and relatively simple preparation. Native β- and γ-CD functionalized columns exhibited satisfactory enantioselectivity for acidic analytes such as dansyl-amino acids. The derivatization of CDs expanded the applicability of the CD-functionalized monolithic CSPs to basic compounds, such as some β-receptor blockers (β-blockers) [54]. However, both enantioselecvity and column efficiency of these columns are still to be further improved.

I.3.1.3. Organic silica hybrid CD monolithic columns

Recently, Zou's group [8] reported a perphenyl-carbamoylated β-CD-silica (Ph-β-CD-silica) hybrid monolithic column which was evaluated using 13 racemic standards. The highest α value reached 1.95, which is much higher than the previously reported value of 1.28 on conventional particulate counterparts.

I.3.1.4. Zirconia-based CD monolithic columns

Zirconia-based [7, 38] β-CD-functionalized monoliths have also been introduced. In Hong and Park's study, sulfated β-CD was dynamically coated on a zirconia-based monolith (ZM) within fused silica capillaries, and six basic compounds were baseline enantioseparated in 20 min [7]. The Rs value for atropine was as high as 8.61.

The current researches about the CD-functionalized monoliths are still mainly focused on improving preparation methods. Although these CD-functionalized monolithic CSPs have shown enantioselectivity towards a broad range of enantiomers, very few studies really aimed at developing applications, particularly in real samples.

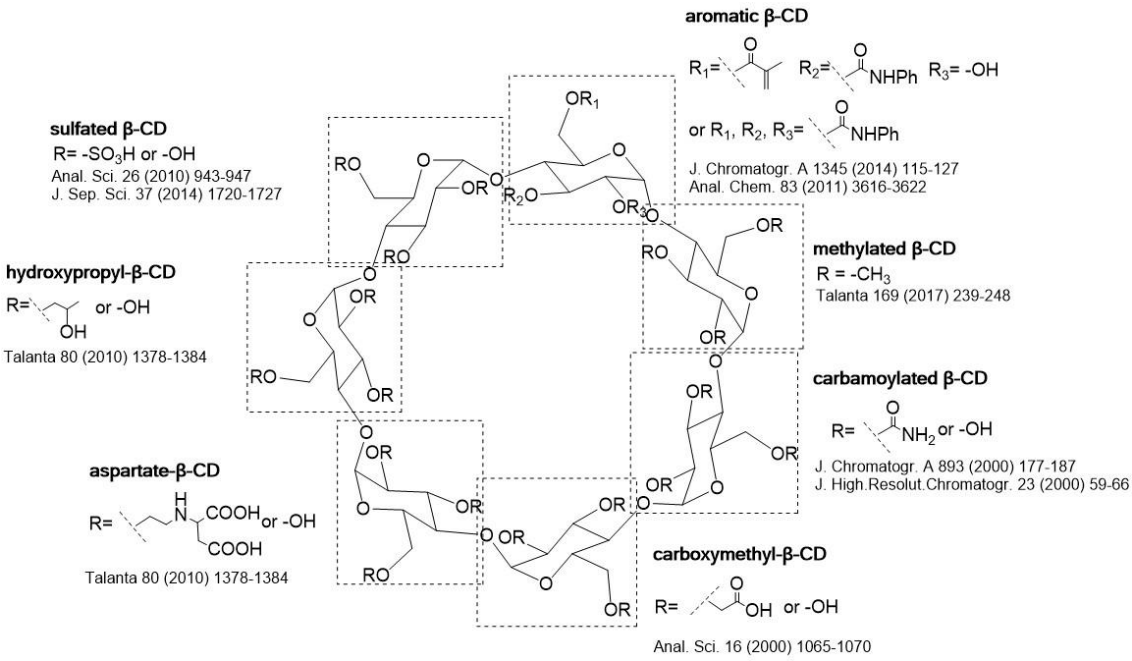


Fig. 6. Structure of β-CD derivatives for monolithic CSPs.

I.3.2. Polysaccharide

Usually, polysaccharide refers to cellulose and amylose derivatives, and they have been most widely used as chiral selector for enantioseparation [55-57]. As far as we know, the development of polysaccharide-functionalized monolithic CSPs was carried out mainly by three groups, i.e. Chankvetadze and Okamoto and coworkers [55, 56, 58-62], Zou *et al*. [57, 63-65], and Park *et al.* [66-68]. However, the poor enantioselectivity of native polysaccharides limited their applications to very polar compounds (such as amino acids). Therefore, great efforts were directed to the development of monoliths functionalized with polysaccharide derivatives in recent decades (**Table 1**), such as amylose *tris*(3,5-dimethylphenylcarbamate) (ADMPC) [69], cellulose tris(3,5-dimethylphenylcarbamate) (CDMPC) [57], cellulose tris(3,5-dimethylphenylcarbamate) derivative [59], and amylose 2,3-(3,5-dimethylphenylcarbamate)-6-ethylphenylcarbamate [70].

I.3.2.1. Silica-based polysaccharide monolithic columns

Okamoto and coworkers [61] first reported the preparation of a CDMPC-functionalized silica-based monolith *via* the physical adsorption approach in 2003. Baseline HPLC enantioseparation of 2,2,2-trifluoro-1-(9-anthryl)ethanol was achieved with retention times of 7.2 and 18.5 s for the first and second eluting enantiomers. Later, Zou *et al*. [57] prepared another CDMPC-functionalized silica-based monolithic

capillary column through the sol-gel process. Fifteen neutral and basic analytes were baseline enantioseparated with high column efficiency in CEC (the highest value reached 239,200 plates/m) using aqueous and nonaqueous mobile phases. Although these cellulose functionalized silica-based monoliths showed good chemical stability against organic solvents, their chemical instability under extreme pH or temperature are still to be overcome.

I.3.2.2. Polymer-based polysaccharide monolithic columns

So far, only a few organic polymer-based polysaccharide-functionalized monolithic CSPs were reported [70]. Fouad *et al.* [70] first reported that three configurations of amylose 2,3(3,5-dimethylphenylcarbamate)-6-ethylphenylcarbamate (including the R-, S-, and racemic-) were encapsulated into the polymer monolith, and they were used to enantioseparate a series of enantiomers under the normal phase and reversed phase. In a study by Dong *et al.* [64], the CDMPC was used as chiral selector which was coated onto poly(acrylamide-co-*N,N*'-methylene-bisacrylamide) (poly(AA-*co*-MBA)) monolithic column, and it exhibited satisfactory stability under both highly acidic (pH 2.7) and basic (pH 9.7) mobile phases.

I.3.2.3. Organic silica hybrid polysaccharide monolithic columns

Zou *et al.* [71] prepared an organic-inorganic hybrid monolith which has the high ordered 3D skeletal microstructure, because there were large primary and secondary amino groups on the hybrid monolith surface, so it was easily physically coated with CDMPC. In addition to the high stability and efficiency due to the polyhedral oligomeric silsesquioxane-poly(ethylenimine) (POSS-PEI) hybrid monolith, the hybrid monolithic column exhibited astonishing enantioselectivity (most Rs values were higher than 2.24) and column efficiency (the highest value was 77,600 plates/m) in CLC mode when it was coated by the CDMPC chiral selector.

I.3.2.4. Zirconia-based polysaccharide monolithic columns

Zirconia-based monoliths are attractive alternatives to silica as monolithic supports for polysaccharide immobilization. Park *et al.* [66-68] prepared CDMPC functionalized-zirconia based monolith (CDMPC-ZM) through the sol-gel approach and post-modification. This method includes two steps, one is the preparation of the zirconia-based monoliths using PEG, water, acetic acid, 1-butanol of suitable

concentrations and zirconium butoxide, another is coating CDMPC onto the zirconia-based monoliths. Finally, the CDMPC-ZM monolithic column exhibited satisfactory stability in eluents of pH ranging from 2.0 to 12.0, and it enabled the fast enantioseparation of β-blockers in less than 1 min by CEC. However, this CDMPC-ZM monolithic column did not show a high column efficiency for all the analytes under the CEC mode.

I.3.3. Proteins

Protein-functionalized CSPs have gained notable success in the enantioseparation of a wide range of enantiomers due to their multiple binding sites for chiral recognition [72, 73]. Proteins include serum transport proteins (human serum albumin (HSA) [74-76], bovine serum albumin (BSA) [77-80] and α-acid glycoprotein (AGP) [81]), enzymes (lipase B [82], pepsin [83], penicillin G acylase (PGA) [84]), other glycoproteins (ovomucoid (OVM) [79, 85] and avidin (AVI) [86]). Recently, an important review article outlined the classification of protein-functionalized monolithic CSPs according to the type of proteins [72].

I.3.3.1. Polymer-based protein monolithic columns

Among the above-mentioned proteins, serum transport proteins are the most commonly employed chiral selectors [72]. In an earlier work, Hage and coworkers [74] reported a series of HSA-functionalized monolithic CSPs within 4.6 mm i.d. stainless steel columns with PEEK inner liners. It was found that all these monolithic columns exhibited certain enantioseparation ability. The highest Rs and α values for tryptophan were 1.52 and 4.32. Later on, Yao *et al.* [76] also developed HSA-functionalized sub-micron skeletal organic polymer-based monoliths for the enantioseparation of D/L-amino acids. The proposed column showed good applicability to the quantitative analysis of D-tryptophan in urine samples as well as the measurement of DAA oxidase (DAAO) enzyme kinetic constants and analysis of real D-AA samples in the enzyme reaction medium. In order to expand the application range in practice, Xu *et al.* [87] developed a mixed protein-functionalized monolithic CSP by co-immobilizing both HSA and cellulase onto the poly(GMA-*co*-EDMA) monolith. For various tested racemic pharmaceuticals, such as, α- and β-blockers, serotonin-reuptake inhibitors, antihistamines, anticoagulants, and amino acids, the mixed protein-functionalized CSP exhibited a wider application range than the single selectors under the same separation

conditions by CEC. Moreover, the enantioresolution for warfarin was found to be improved.

I.3.3.2. Silica-based protein monolithic columns

Protein-functionalized silica-based monoliths have also been systematically investigated [78-80, 85]. Hage *et al.* prepared HSA [75] and AGP [81]-functionalized silica-based monoliths using a hydrazide immobilization method. There are some significant advantages of this method, such as higher protein coverage, column efficiency and resolution. For example, compared to its particle-based or the poly(GMA-*co*-EDMA) monolith-based counterparts, this HSA-functionalized silica-based monolith showed higher density of immobilized HSA (1.3 to 2.2 times), and therefore higher or comparable enantioresolution and efficiency for D/L-tryptophan and R/S-warfarin. Toyo'oka *et al.* [80] also reported the preparation of BSA or OVM-functionalized silica-based monolithic capillary columns by encapsulating the corresponding protein into silica-based matrix. The BSA-encapsulated monolith exhibited enantioresolution for tryptophan and benzoin, while the enantioseparation of eperisone, chlorpheniramine and benzoin was achieved on the OVM-encapsulated monolith. However, the column efficiency for the second eluting enantiomer was rather low. Massolini and coworkers [88] also developed a series of PGA-functionalized silica-based monoliths for the enantioseparation of ketoprofen, suprofen, fenoprofen, 2-aryloxyalkanoic acid methyl esters, 2-aryloxyalkanoic acids and 2-arylpropionic acids. Such a monolithic column was further applied to the determination of (S) ketoprofen in pharmaceutical samples.

I.3.3.3. Nanoparticles-mixed protein monolithic columns

Some nanomaterials, including graphene oxide (GO) [83], carboxylated single-walled carbon nanotubes (c-SWNTs) [89] and amino-modified mesoporous silica nanoparticles (NH_2-MSNs) [90], were also employed for preparing protein-functionalized monolithic CSPs, in order to increase the surface area and then to improve the enantioseparation performance. GO is a chemically modified graphene sheet with a variety of reactive oxygen functional groups. GO-modified silica-based affinity capillary monoliths employing HSA or pepsin as chiral selectors [83] were recently developed, and the resulting monoliths exhibited higher chiral selectivity for different pairs of enantiomers, such as tryptophan, phenylalanine, azelastine, warfarin,

ibuprofen, salbutamol, chlortrimeton, propranolol, ritodrine and nefopam, compared to the columns without GO. Ji *et al.* [89] developed c-SWNT modified pepsin based polymeric monoliths. In comparison with the monoliths without c-SWNTs, stronger retention (for nefopam, 3.49 min on the c-SWNT-incorporated column vs 1.50 min on the control column) and higher separation efficiency (**Fig. 7**) were achieved on the c-SWNT-incorporated columns. The resulting monolith exhibited excellent enantioseparation performance for ten pairs of basic drugs. Similar advantages were also found for the NH_2-MSN incorporated pepsin based hybrid monoliths [90]. The amino modified mesoporous silica nanoparticles provided additional interaction sites, and consequently yielded a different enantioselectivity and an enhancement of the overall separation factors. Compared to the control column without NH_2-MSNs, enantioresolution values were significantly improved on this column. Nine basic chiral drugs could be baseline enantioresolved and six could be partially enantioseparated within 10 min using aqueous mobile phases. The highest resolution factor (Rs) was 3.24 for clenbuterol enantiomers.

The broad applicability of the protein-functionalized monolithic CSPs is evident from the number of racemates enantioseparated, but there are still some shortcomings that need to be overcome, such as unsatisfactory affordability and disposability, as well as the low immobilization efficiency of the proteins. Further research is expected to improve the stability of these monolithic CSPs, prolong their lifetime and simplify the functionalization procedures.

I.3.4. Antibiotics

Armstrong and co-workers first introduced macrocyclic antibiotics as chiral selectors in liquid chromatography in 1994 [91]. In recent years, various classes of antibiotic-functionalized monolithic CSPs, with vancomycin, teicoplanin, norvancomycin, azithromycin, clindamycin phosphate (CLIP), and erythromycin as chiral selectors, have been successfully developed for enantioseparation in CEC or CLC (**Table 1**). As far as we know, Gübitz *et al.* [92] and Maruška *et al.* [93] were the pioneers in the field of antibiotic-functionalized monolithic CSPs.

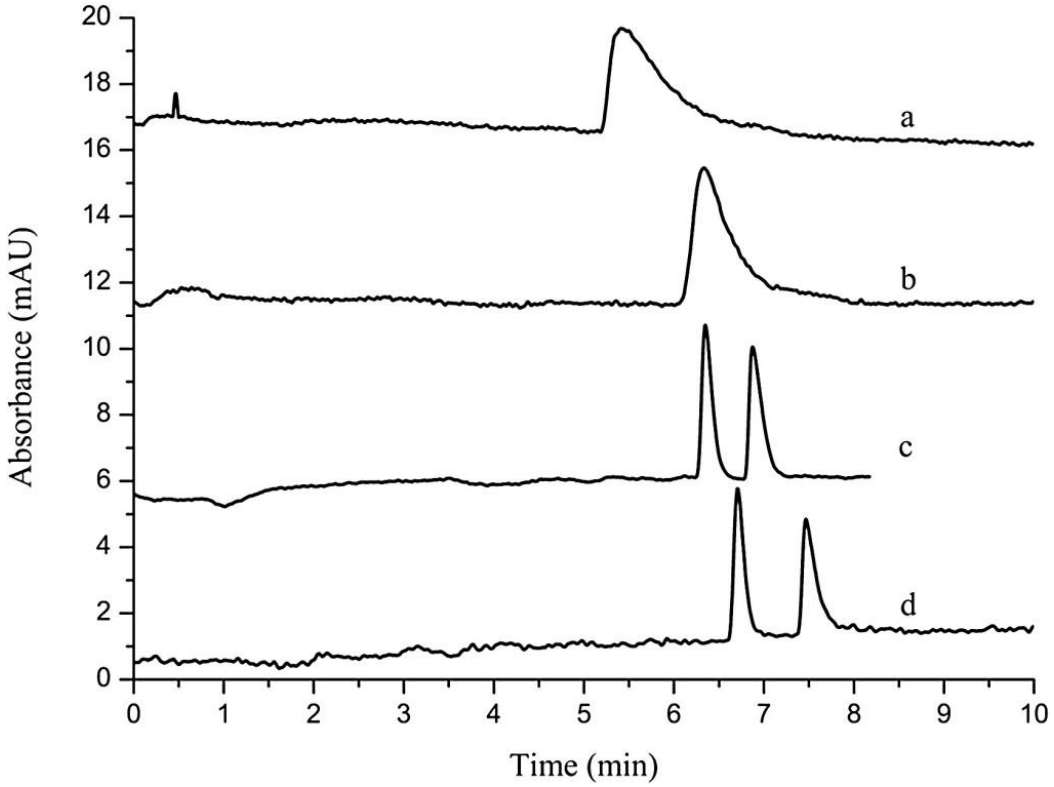


Fig. 7. Influence of the incorporation of c-SWNTs and pepsin in the polymer monolith on the enantioseparation of (±)-nefopam. (a) poly(GMA-*co*-EDMA) monolith; (b) poly(GMA-*co*-SWNT-EDMA) monolith; (c) pepsin-immobilized poly(GMA-*co*-EDMA) monolith; (d) pepsin-immobilized poly(GMA-*co*-SWNT-EDMA) monolith [89].

I.3.4.1. Polymer-based antibiotic monolithic columns

Vancomycin, an amphoteric glycopeptide mainly produced by Streptomyces orientalis or Amycolatopsis orientalis, is one of the most frequently studied chiral selectors for antibiotics-functionalized monolithic CSPs. The first report on the fabrication of vancomycin-functionalized organic polymer-based monoliths was published by Maruška and coworkers [93]. Vancomycin was immobilized onto poly(*N*-(hydroxymethyl)acrylamide-*co*-piperazine diacrylamide) by reductive amination. Baseline enantioseparation was obtained in CEC for thalidomide, while the other three racemic compounds, i.e. warfarin, coumachlor and felodipine, were partially enantioseparated [93]. Later on [94], another vancomycin-functionalized polymeric monolith was prepared through a simpler approach *via* reductive amination of the aldehyde groups on the polymeric skeleton, and this monolithic column was evaluated using a set of enantiomers by reversed phase CEC; however, the enantioseparation results were unsatisfactory.

I.3.4.2. Silica-based antibiotic monolithic columns

Other types of supports, especially vancomycin-functionalized silica-based monolithic CSPs were also studied [95-97]. Hsieh *et al.* [96] recently developed a single-step *in situ* sol-gel approach to prepare vancomycin functionalized silica-based monolith. First, the precursor containing vancomycin was synthesized by sol-gel, then it was co-polymerized with skeleton precursor to form a porous silica-based monolith. This monolithic column not only exhibited good efficiency and enantioselectivity for many basic enantiomers, but also significantly simplified the preparation procedure. In addition, Pittler and Schmid [97] also reported a dynamic coating method to prepare monolithic CSPs functionalized with *N*-(2-hydroxydodecyl)-derivative of vancomycin (a hydrophobic alkyl chain was attached to the vancomycin molecule). A series of dansyl amino acids were enantioresolved under RP-HPLC mode. Interestingly, a reversal of the enantiomer (dansyl amino acids) elution order was observed on this type of column.

Norvancomycin, another glycopeptide antibiotic with similar structure as vancomycin, has also been used as chiral selector. The preparation and application of norvancomycin-functionalized monolithic CSPs were mainly carried out by Ding's group [98, 99], and the enantioseparation of thalidomide in real samples was achieved using CEC.

I.3.4.3. Zirconia-based antibiotic monolithic columns

Zirconia-based monoliths (ZM) have also been introduced to further improve the stability. Park *et al.* [100] reported the fabrication of a CLIP-functionalized ZM for the enantioseparation of acidic and basic drugs using CLIP-saturated polar organic mobile phases. No appreciable declines in resolution and retention were observed after over 50 injections, and the run-to-run and day-to-day reproducibilities for this column were less than 3%. More recently, a series of antibiotics including CLIP, erythromycin, and azithromycin were successfully incorporated into organic-zirconia hybrid monoliths (ZHMs) through a single-step *in situ* sol-gel approach [101-103]. These organic-ZHMs not only showed an excellent stability in mobile phases of pH > 10, but also exhibited good enantioseparation ability for several chiral acidic drugs, such as ketoprofen, carprofen, flurbiprofen, ibuprofen, suprofen and warfarin [103]. Their superior separation performance and efficiency could be attributed to the homogenous distribution of the organic moiety in the 3D inorganic matrix.

I.3.5. Ligand-exchange (LE)

Chiral Ligand-exchange (LE) type chromatography (CLEC) was first proposed by Davankov [104], it represents a widely used chiral separation principle successfully used to enantioresolve racemates in HPLC, CE and CEC [105, 106]. To the best of our knowledge, Schmid, Gübitz *et al.* [105], Chen, Hobo *et al.* [107], and Mizrahi, Lev *et al.* [108] are the three main groups in the field of LE monolithic CSPs.

I.3.5.1. Polymer-based LE monolithic columns

Polymer-based LE monolithic columns were mainly reported by Schmid and coworkers from 2000 to 2010 [109-112]. First, they prepared a chiral LE CSP based on continuous bed by a one-step in 2000, the chiral selector (*N*-(2-hydroxy-3-allyloxypropyl)-L-4-hydroxyproline) was introduced into the polymer continuous bed by copolymerization with methacrylamide, piperazine diacrylamide, vinylsulfonic in 0.05 M phosphate buffer (pH 7-8) [109]. Later on, L-prolinamide and L-4-hydroxyproline derivatives were introduced into the polymer continuous bed by the same strategy. Although some amino acids and hydroxyl acids were separated using these monoliths, the efficiency was unsatisfactory [110-112].

I.3.5.2. Silica-based LE monolithic columns

Schmid *et al.* [113] used *N*-decyl-L-4-hydroxyproline, *N*-hexadecyl-L-4-hydroxyproline and *N*-2-hydroxydodecyl-L-4-hydroxyproline as chiral selector to dynamically coate onto commercially available silica-based RP-18 monolithic columns. Interestingly, 23 amino acids and 3 dipeptides could be baseline separated on this type of CSP with acceptable column efficiency. Furthermore, Chen *et al.* [107, 114-117] also prepared a series of LE type silica-based monolithic CSPs. First, they prepared a silica-based monolithic column matrix using acetic acid, PEG and TMOS by a sol-gel process. Then, the spacer (3-glycidoxypropyltrimethoxysilane (3-GPTM)) and the chiral selector was sequentially modified onto the surface of the silica-based monolithic column matrix. So far, L-phenylalaninamide (L-PheA) [107, 114, 116], L-prolinamide (L-ProA), L-alaninamide (L-AlaA) [115, 116] and L-hydroxyproline [117] were used as chiral selectors onto the surface of silica-based monolithic column matrix.

I.3.6. Ion-exchange (IE)

There are a lot of choices for ion-exchange (IE) chiral selectors, such as chiral anion-exchangers (terguride and cinchona alkaloid derivatives), chiral cation exchangers (amino sulfonic and carboxylic acids) and zwitterionic ion-exchangers (which merged the structural elements of above anion and cation exchangers) [118]. However, cinchona alkaloid derivatives were the most widely used due to the great contributions from Lämmerhofer's group [119, 120].

I.3.6.1. Polymer-based IE monolithic columns

Lämmerhofer *et al.* [11, 121-125] reported polymer-based IE monolithic columns using quinine and quinidine derivatives as the chiral selectors. This simple single-step strategy just needed to mix the monomer (MQD, GMA) or 2-hydroxyethyl methacrylate (HEMA)), the cross-linker (EDMA), the initiator AIBN and the porogenic solvent. Then, the polymer monoliths were prepared by photo-initiation or thermal-initiation, and they exhibited good enantioseparation potential for amino acids derivatives, mecoprop, and fenoprop. The effect of the co-monomers GMA and HEMA was investigated and the results showed that when GMA was used as the co-monomer, the monolithic column pore size was smaller than when using HEMA, while the column efficiency was lower than for HEMA. In order to further study the enantioseparation with these chiral selectors [123], cinchona alkaloid derivatives including *O*-9-(*tert*-butylcarbamoyl)-11-[2 (methacryloyloxy)ethylthio]-10,11-dihydroquinine and *O*-9-[2-(methacryloyloxy)ethylcarbamoyl]-10,11-dihydroquinidine were developed, showing that the chiral selector which had the bulky carbamate residue exhibited higher enantioresolution. Furthermore, they also prepared a *O*-9-tert-butylcarbamoylquinine(*t*-BuCQN) functionalized IE monolithic column by radical addition of *t*-BuCQN to the thiol groups [124], and it was proven that thiol-modified can be used for the preparation of the monoliths.

Recently, in order to broaden the application range of quinidine-functionalized monoliths, the MQD-functionalized organic polymer-based monolith according to Lämmerhofer *et al.* [11] was systematically re-optimized by Jiang *et al.* [126]. The resulting monoliths exhibited good enantioselectivity for most of the studied *N*-derivatized amino acids [126], *N*-derivatized small peptides [127], and herbicides [128] in micro-LC mode. The enantiomeric purity testing of R-mecoprop [128] and L-carnosine in commercial samples was also carried out on this monolithic column [127].

It was found that the monolith could be used as a reliable tool for the quality control of these compounds in commercial samples by micro-LC. The influence of the co-monomer HEMA on the enantioseparation ability was also investigated by comparing the enantioselectivities of poly(MQD-*co*-HEMA-*co*-EDMA) and poly(MQD-*co*-EDMA) monoliths [129]. For *N*-benzoylated and FMOC-derivatized amino acids, similar enantioselectivities were observed on both monoliths, which proves that the effect of the co-monomer HEMA on enantioselectivity in quinidine based monoliths is not crucial. Furthermore, by using a self-assembled laser induced fluorescence (LIF) detector, the poly(MQD-*co*-EDMA) monolithic column could be used for determining traces of D-amino acids (fmol level) in the presence of large amounts of their L-forms [129]. Inspired by the *t*-BuCQN-functionalized monolith, the *O*-9-(*tert*-butylcarbamoyl)-11-[2-(methacryloyloxy)ethylthio]-10,11-dihydro-quinidine (MBQD) -functionalized monolithic column was developed to enhance the enantioselectivity of the quinidine (MQD) -functionalized monolithic column [130]. It is worth noting that this monolithic CSP containing quinidine with a *tert*-butyl carbamate residue as chiral selector exhibited much higher enantioselectivity and diastereoselectivity for *N*-derivatized amino acids and dipeptides than the previously developed MQD-functionalized monolithic CSP (**Fig. 8**) [127].

Besides the cinchona alkaloid derivatives, a series of other chiral selectors including (S)-*N*-(4-allyloxy-3,5-dichlorobenzoyl)-2-amino-3,3-dimethylbutane phosphonic acid [128] and leucine-based carbamate chiral surfactants (sodium 8-acrylamidooctenoxy carbonyl-L-leucinate (SAAOCL), sodium 10-acryl-amidodecenoxy carbonyl-L-leucinate (SAADCL), and sodium 12-acryl-amidododecenoxy carbonyl-L-leucinate (SAADoCL)) [131], were also used for the preparation of chiral IE monolithic columns by different technologies, and applied for the enantioseparation of mefloquine, mefloquine *tert*-butylcarbamate and ephedrine, pseudoephedrine, respectively.

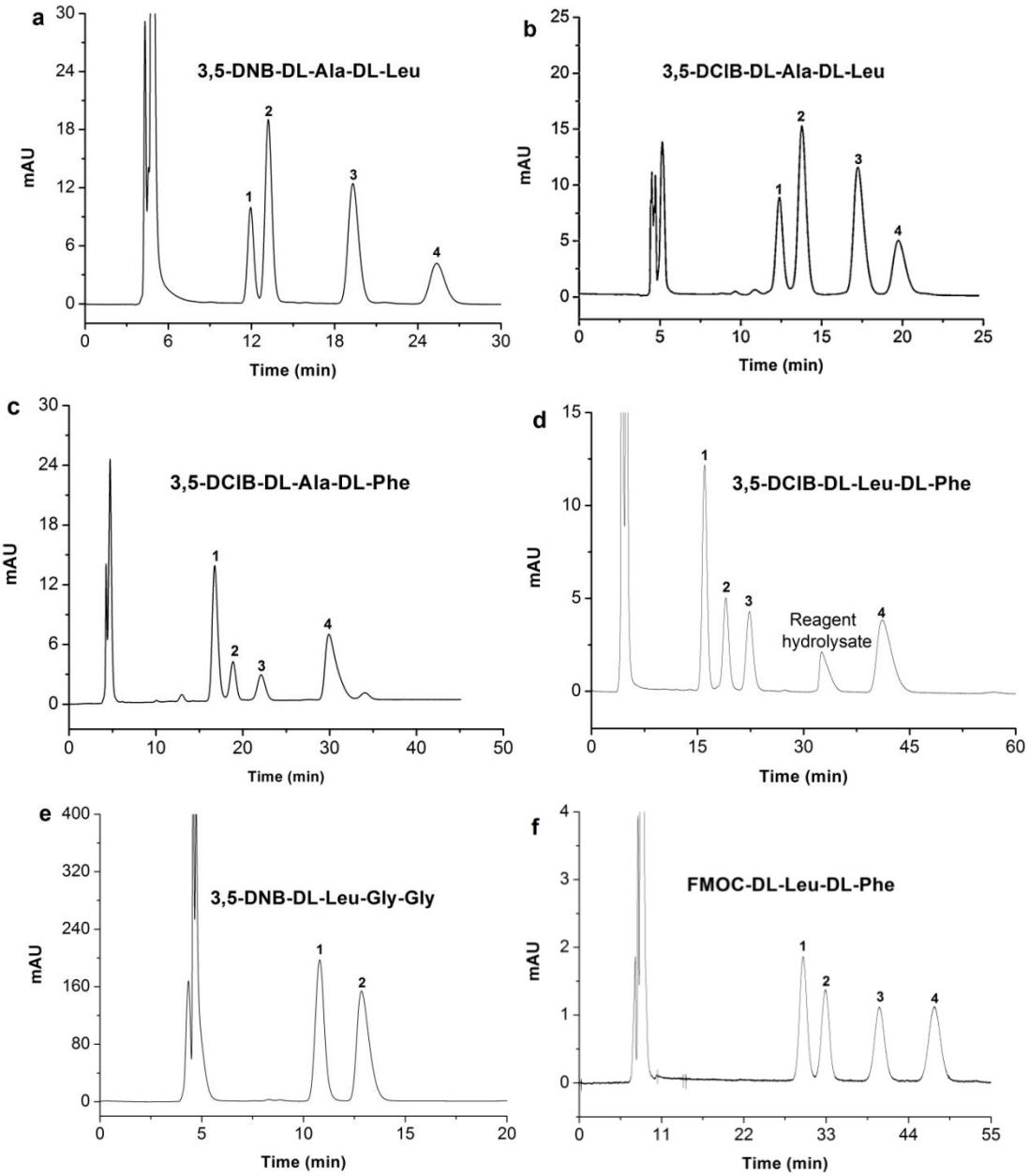


Fig. 8. Separation of *N*-derivatized small peptide stereoisomers on a poly(MQD-*co*-HEMA-*co*-EDMA) monolithic column [127].

I.3.6.2. Silica-based IE monolithic columns

The cinchona alkaloid derivatives were also common chiral selectors for the fabrication of silica-based IE monolithic column. Lubda *et al.* [132] prepared silica based monoliths modified with chiral selectors by two steps: (i) preparation of a silica monolith column modified with mercaptopropyl groups (-SH), then (ii) the chiral selector (*tert*-butylcarbamoylquinine (*t*-BuCQN) or *O*-9-(*tert*-butylcarbamoyl)quinidine (*t*-BuCQD)) was introduced into the surface of the above silica monolith by thiol-ene click chemistry. Finally, they were successfully used for

the enantioseparation of amino acid derivatives and four stereoisomers of phosphinic acid-derived pseudodipeptides. Afterwards, Buchinger *et al.* [133] prepared another *t*-BuCQD functionalized silica-based monolithic column by three steps: (a) preparation of a silica monolith column modified with glycidoxypropyl trimethoxysilane, (b) the epoxy-modified surface reacted with hydrogen sulfide to introduce active sulfhydryl groups as anchors, and (c) the chiral selector *t*-BuCQD was introduced into the surface of the silica monolith by thiol-ene click chemistry. Finally, good enantioseparation was obtained for 2-aryloxypropionic acid herbicides including dichlorprop, mecoprop and fenoprop.

Additionally, some analogous silica-based IE monolithic columns containing (S)-*N*-(4-allyloxy-3,5-dichlorobenzoyl)-2-amino-3,3-dimethylbutane phosphonic acid [134] and *trans*-(1S,2S)-2-(N-4-allyloxy-3,5-dichlorobenzoyl)amino cyclohexane sulfonic acid [135, 136] as chiral selectors, also exhibited good enantioseparation for amino alcohol drugs in CEC or CLC.

I.3.6.3. Organic silica hybrid IE monolithic columns

Tran *et al.* [137] prepared a quinine functionalized silica/zirconia hybrid monolith through a conventional sol-gel process of 3-triethoxysilylpropylcarbamated quinine and zirconium tetrabutoxide in solution containing polyethylene glycol (PEG), acetic acid, *n*-butanol and water. The obtained hybrid monolith exhibited good enantioselectivity for profens and dinitrobenzoyl (DNB) amino acids in CEC mode. In another work [138], they also prepared quinine functionalized silica hybrid monoliths *via* both sol-gel chemistry and free radical polymerization of *t*-BuCQUI, vinyltrimethoxysilane (VTMS) and TMOS. More recently, cinchona alkaloid-functionalized hybrid monoliths were also prepared by photo-initiated thiol-ene polymerization [139] and used for the enantioseparation of profens, tropic acid, warfarin, mandelic acid, or *N*-derivatized amino acids, etc.

I.3.7. Brush-type

Brush-type (or Pirkle-type) CSPs, consisting of small organic molecules covalently bonded to the support, are also one of the most widely investigated CSPs [140]. The chiral recognition mechanism of these CSPs is based on different interactions between the analyte and the chiral selector: dipole-dipole interactions, hydrogen bonds, van der Waals interactions as well as aromatic interactions.

I.3.7.1. Silica-based Brush-type monolithic columns

The first Brush-type monolithic CSPs were reported by Kato and his colleagues [141]. First, two types of silica particles were modified with the chiral selector ((S)-*N*-3,5-dinitrobenzoyl-1-naphthylglycine or (S)-*N*-3,5-dinitrophenylaminocarbonyl-valine), then the above modified silica particles embedded in the silica-based monolith through sol-gel method. A series of 7-nitro-2,1,3-benzoxadiazole (NBD)-derivatized amino acids and non-protein amino acids were separated in CEC.

It is worth noting that "Brush-type" silica-based monoliths received more and more attention in recent years. Slater and co-workers [142] first introduced the azide functionalities into the surface of silica-based monoliths by reaction with 3-(azidopropyl)trimethoxysilane; then, the chiral selector (*N*-prolinoyl-5-aminoindan) was immobilized onto the monolith by click chemistry. Later, Sancho *et al.* [143] prepared silica-based monolithic columns by covalently bonding two different poly-(4R)-(3,5- dimethylphenylaminocarbonyloxy)-L-proline derivatives by reaction between the primary amino group (modified monolith-NH_2) and carboxyl group (chiral selector-COOH). Based on the above preparation strategies, Novell *et al.* [144, 145] used the non-substituted-octaproline and poly-(4R)-(3,5-dimethylphenylaminocarbonyloxy)-L-proline derivatives as chiral selector bonded onto the silica particles and silica-based monoliths to compare their chromatographic behaviour and performance. The results showed that silica-based monolithic columns had better performance than silica particle packed columns. In addition, Ghanem *et al.* [146] prepared a new silica-based monolith bonding a single low-molecular mass chiral selector (namely (R)-acryloyloxy-β-β-dimethylg-butyrolactone) *via* in-situ polymerization. Finally, a set of secondary alcohols were baseline separated by CLC.

I.3.7.2. Polymer-based Brush-type monolithic columns

Recently, Jiang *et al.* [147] reported the preparation of *N*-[1-(α-naphthyl)ethylaminocarbonyl]-D-tert-leucine-[2-(methacryloyloxy)ethyl] amide-functionalized organic polymer-based monoliths *via* single step thermo-initiated copolymerization or multi-step post-modification methods. The monolith prepared through the latter approach showed much higher enantioselectivity for NBD-derivatized amino acids in micro-LC than that fabricated *via* the former method. This may be attributed to the fact that a certain amount of chiral selector was buried into the

polymer skeleton during the single step copolymerization [148].

I.3.8. Molecularly imprinted monolithic columns

Molecular imprinting (MI) is a widely used technique. It can achieve specific recognition at a molecular level due to the construction of a synthetic polymer receptor with tailor-made binding sites [149, 150]. In recent years, chiral molecular imprinting monolithic columns based on the MI technique have also been developed. Because of the good stability at high pressure, temperature, and strong acids and bases, these columns have received more and more attention [151-155]. The preparation method of chiral MI monolithic columns is similar to that of the above polymer or silica-based monolithic columns, even the approach of the chiral MI monolithic columns is simpler, because it does not require the derivatization of the chiral selector. Yin *et al.* [156] prepared polymer-based MI monolithic columns by mixing the chiral template (nateglinide), the monomer (acrylamide), the crosslinker (EDMA) and the porogenic solvents (cyclohexanol and 1-dodecanol). Then, the chiral template was completely removed using acetic acid/methanol, methanol, methanol/triethylamine and ACN. Finally, this chiral MI monolithic column enabled the enantioseparation of nateglinide and its L-enantiomer, whereas this enantioseparation was not obtained on the non-imprinted polymer column. He *et al.* [157] prepared silica-based chiral MI monolithic columns by three steps: (a) preparation of a silica-based monolith by the sol-gel method, (b) modification of the silica-based monolith by γ-methacryloxypropyltrimethoxysilane (γ-MAPS), (c) mixing the template, monomer (methacrylic acid), EDMA, AIBN and toluene/ACN solvent, and injecting into the silica-based monolithic column. In addition, molecular crowding-based imprinted monolithic columns were developed [158-160]. Zong *et al.* [158] and Wang *et al.* [160] used *d*-zopiclone and S-amlodipine as the chiral templates mixed with MAA, EDMA, AIBN and macromolecular crowding agent (poly(methyl methacrylate)) (PMMA) into $CHCl_3$ solvent to prepare polymer-based chiral molecular crowding-based imprinted monolithic columns. They exhibited good separations for L-zopiclone and racemic amlodipine, respectively, as shown in **Fig. 9**.

However, chiral MI monolithic columns also face many problems. For example, the preparation approach of silica-based chiral MI monolithic columns was complicated and time-consuming while organic polymers were easily shrinking or swelling in organic solvents, and most importantly, their low column efficiency greatly limited their application in the field of chromatographic separation analysis.

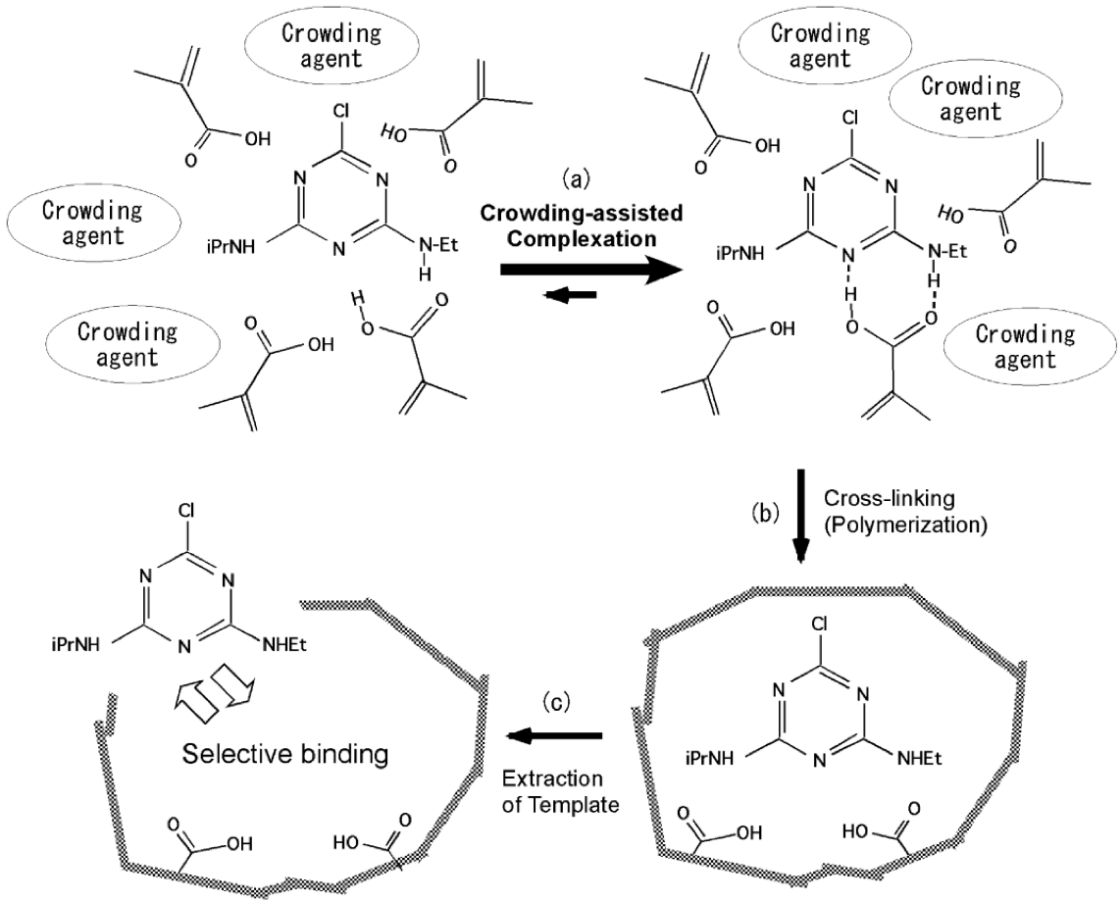


Fig. 9. Schematic representation of molecular imprinting under molecular crowding conditions. Atrazine (a template molecule) is mixed with MA (a functional monomer) in chloroform with PMMA or PS as a macromolecular co-solute (a); cross-linking with EDMA is achieved by heating, forming a three-dimensional polymer matrix (b); atrazine is extracted from the matrix to leave behind a binding site (c) [159].

Table 1. Different chiral selector-functionalized monolithic columns and their matrices.

Classification	Polymer	Silica	Hybrid	Zironia
CD	β-CD [9, 40, 42, 48, 52]; 4-dimethylamino-1,8-naphthalimide-β-CD [45]; hydroxypropyl-β-CD [9]aspartate-β-CD [9]	β-CD [161, 162]; Perphenylcarbamoylated β-CD [6]; sulfated β-CD [18]	Perphenylcarbamoylated β-CD [8]	Sulfated β-CD [7]; phosphated β-CD [38]
Polysaccharide	Cellulose tris(3,5-dimethylphenylcarbamate) [64]	Cellulose tris(3,5-dimethylphenylcarbamate) [57, 63, 65, 163]; amylose tris(3,5-dimethylphenylcarbamate) [69]	Amylose tris(3,5-dimethylphenylcarbamate) [58, 71]	Cellulose dimethylphenylcarbamate [65-68]
Protein	HAS [74]; AGP [81]; lipase [82]; pepsin [89]	HAS [75, 83, 164]; BSA [77-80, 165]; AGP [81]; OVM [80, 85]; avidin [166]; pepsin [167]; PGA [84]	Pepsin [90]	/
Antibiotic	Vancomycin [93, 94, 168]	Teicoplanin [92]; teicoplanin aglycone [169]; vancomycin [95-97]; norvancomycin [98, 99];	Clindamycin phosphate [101]; erythromycin [102]; azithromycin [103]	/

Ligand-exchange	L-4-hydroxyproline and its derivatives [105, 109-112]; L-prolinamide [111]; D-valine [108]	L-4-hydroxyproline and its derivatives [113, 117]; L-prolinamide and L-alaninamide [107, 115, 116]; L-phenylalaninamide [114]	/	/
Ion-exchange	*O*-9-[2-(methacryloyloxy)ethylcarbamoyl]-10,11-dihydroquinidine [11, 121-123, 126-129]; *O*-9-*tert*-butylcarbamoyl quinine [120, 124]; *O*-9-(tert-butylcarbamoyl)-11-[2-(methacryloyloxy)ethylthio]-10,11-dihydroquinidine [130]; *O*-9-(tert-butylcarbamoyl)-11-[2 (methacryloyloxy)ethylthio]-10,11-dihydroquinine [123]; (S)-*N*-(4-allyloxy-3,5-dichlorobenzoyl)-2-amino-3,3-dimethylbutane phosphonic acid [128]; leucine-based carbamate chiral surfactants [132]	*O*-9-*tert*-butylcarbamoyl quinine [131, 133]; (S)-*N*-(4-allyloxy-3,5-dichlorobenzoyl)-2-amino-3,3-dimethylbutane phosphonic acid [134]; *trans*-(1S,2S)-2-(*N*-4-allyloxy-3,5-dichlorobenzoyl) aminocyclohexanesulfonic acid [135, 136]	*O*-9-[2-(methacryloyloxy)ethylcarbamoyl]-10,11-dihydroquinidine [170]; *O*-9-*tert*-butylcarbamoyl quinine [137, 138]; phenylisocyanate cinchonidine [139]	/

Brush-type	(*S*)-*N*-3,5-dinitrobenzoyl-1-naphthyl-glycine and (*S*)-*N*-3,5-dinitrophenylaminocarbonyl-valine [141]; *N*-[1-(*α*-naphthyl)ethylaminocarbonyl]-D-*tert*-leucine-[2-(methacryloyloxy)ethyl] amide [147];	(*R*)-α-acryloyloxy-β,β-dimethyl-γ-butyrolactone [146]; *N*-prolinoyl-5-aminoindan [142]; poly-(4*R*)-(3,5-dimethylphenylaminocarbonyloxy)-L-proline derivatives [143, 145]; octaproline [144]	/	/

I.4. Preparation strategies

For the preparation of monolithic CSPs, the choice of a suitable fabrication approach is decisive not only for repeatability and physicochemical properties (pore size distribution, mechanical and chemical stability, morphology, column efficiency etc.), but also for enantioselectivity owing to the way of the chiral selectors is attached to the monolithic matrix and in which amount. Therefore, great efforts have been contributed to improve the preparation efficiency since the development of monolithic CSPs. To date, several approaches including particle fixing [171, 172], encapsulation [78-80, 173-175], physical adsorption [21] and covalent bonding [63, 176], have been employed for preparing monolithic CSPs.

Wistuba *et al.* [21, 22, 161] first reported particle fixing approaches for embedding ChiraDex (permethyl-β-CD) particles in a silica-based continous bed by sintering at high temperature [21] or gluing *via* a sol-gel process [22]. The particles were first packed into a capillary and then subjected to a fixing procedure. The resulting columns showed good enantioseparation ability for selected compounds in both CEC and CLC. However, packing particles into a capillary as a first setp is still a time and skill-demanding task. Moreover, these authors found that the ChiraDex-glued column exhibited lower enantioresolving power than its particulate counterpart, and they ascribed this to the fact that the incorporation of the CD rings within the silica-network may partially shield the interactions between the chiral selectors and racemic analytes. No further studies were carried out. A modified version of the particle fixing approach was reported by Schmid [92] for preparing teicoplanin glycone functionalized monolithic CSPs. The chiral silica particles were first suspended in the polymerization mixture. After adding the initiator, the solution was immediately sucked into a capillary for the copolymerization and embediment of the chiral silca particles. Although the columns exhibited good enantioseparation for a series of amino acids and dipeptides, the column efficiency was not satisfactory, and this may be due to poor dispersion of the silica particles in the polymerization mixture since these materials are prone to settling when used in high percentages.

Encapsulation is a notable approach for enzyme immobilization due to its simplicity [177]. It has been applied to the preparation of monolithic CSPs functionalized with proteins (e.g. BSA [79], OVM [80] and lipase [82]), macrocyclic antibiotics [175] or CDs [174]. These chiral selectors can be easily entrapped in the monolithic matrix during *in situ* copolymerization or a sol-gel process, avoiding the difficulties of

derivatization. Although the encapsulation approach is straightforward and the entrapped chiral selectors are supposed to maintain their enantio-recognition abilities, the reported enantioseparations based on these monolithic CSPs have so far not been very satisfactory [80]. In addition, the leakage of the chiral selector from the matrix as well as the column reproducibility, are still issues to be solved when applying the encapsulation approach.

Thanks to the large amount of available knowledge on silica gel modification, physical adsorption [166] or covalent bonding [63, 176] have become more popular approaches nowadays for introducing chiral selectors onto the surface of monoliths. The *in situ* physical adsorption is a kind of post-modification approach whereby the chiral selector is attached to the monolithic matrix as a result of hydrogen bonding, van der Waals forces, electrostatic forces, hydrophobic interactions etc. In principle, it provides a simple, fast and more universal way for introducing diverse chiral selectors into the monolithic matrix (e.g. proteins [166], cellulose derivatives [178], ligand-exchangers [113] and β-CD and derivatives [19, 20]). However, the adsorbed chiral selectors often showed lower chemical stability, which limited the selection of suitable mobile phases. The *in situ* physical adsorption may also create an inhomogeneous film of chiral selectors, which is unfavorable for peak shape and efficiency. Recently, more effort has been directed towards the covalent bonding approach because it can produce monolithic CSPs with better chemical stability, a wider application range and even higher column efficiency. Chiral selectors could be covalently bonded onto the matrix mainly *via* an amino linkage [8, 41, 44, 45], ether linkage [36], or triazole linkage [40, 42, 43] etc. Pioneering work for the covalent bonding of chiral selectors was reported by Hjerten's group in 1993 [179]. Cellobiohydrolase I from *Trichoderma reesei* was immobilized onto organic polymer-based matrices through either a ring-opening reaction of an epoxy-activated polymeric bed or one-step copolymerization of allyl cellulase. Both types of columns showed good enantioseparation performance for β-blockers in terms of speed and resolution. The enantiomers of practolol were baseline resolved within 45 sec, which highlighted the advantage of fast separations with monolithic CSPs. More recently, the first click-derived CSP was reported by Kacprzak *et al.* [180-182]. "Click chemistry" (including alkyne-azide, thiol-ene, and thiol-yne reactions) can orientatedly immobilize chiral selectors onto a monolithic matrix due to its high efficiency, high selectivity, and mild reaction conditions [144]. This further enriched and improved the covalent bonding technologies. This section will mainly

focus on three different covalent immobilization approaches for monolithic CSPs, i.e. post-modification, single-step, and one-pot approaches, but will not deal in detail with general aspects of monolith preparation.

I.4.1. Post-modification approah

Post-modification might be the most versatile approach for covalently bonding chiral selectors. A monolithic matrix with proper pore structure as well as active functional groups is first prepared *via in situ* copolymerization or sol-gel process; a chiral selector is then immobilized through designated chemical reactions with these active moieties on the surface (**Fig. 10**). This multi-step approach could effectively separate the control of column morphology from the manipulation of enantioselectivity. In a recent review, Jandera and coworkers summarized well the advantages of this approach and the chemical reactions used for the post-modification of organic polymer-based monolithic columns [183]. They highlighted the fact that the poly(GMA) and poly(*N*-acryloxysuccinimide) (poly(NAS)) monoliths are probably the most commonly used reactive polymers for post-modification.

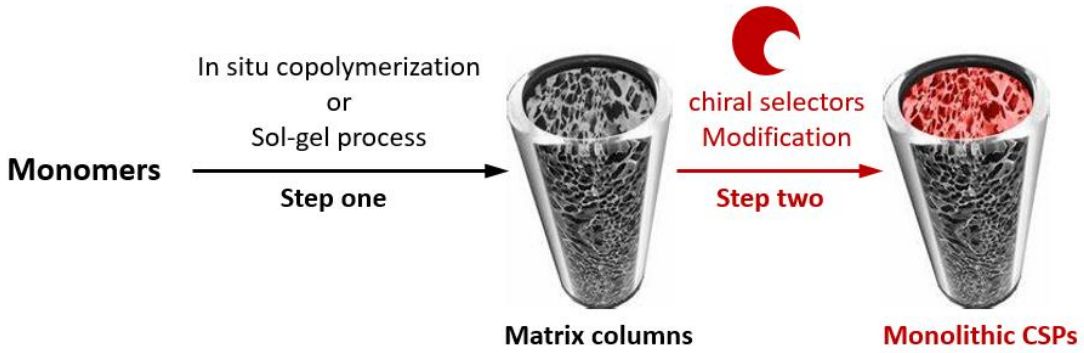


Fig. 10. Scheme for the preparation of monolithic CSPs *via* a post-modification approach.

The epoxy group has a highly reactive functionality which can react with nucleophiles (e.g. primary amines, sulfhydryls, or hydroxy groups) during a ring opening process. Therefore, the epoxy-containing monomers (e.g. GMA [124]and allyl glycidyl ether (AGE) [93, 94]) were often used to generate an epoxy-activated monolithic polymers, while epoxy-activated silica monoliths were produced by *in situ* activation of monolithic silica with γ-glycidoxypropyl trimethoxysilane [54, 75, 81]. Subsequently, chiral selectors could directly and indirectly react with these epoxy groups under mild conditions.

The epoxy groups could react directly with the chiral selectors containing primary

amines, hydroxy groups or other nucleophiles [41, 114, 115, 184] (**Fig. 11a and b**). This direct post-modification method was commonly applied for immobilizing different chiral selectors onto the epoxy-activated silica-based monolith [6, 18, 59, 84], such as amino acids and their derivatives [115, 117], proteins [185], cellulose derivatives [59], etc. For example, Chankvetadze *et al.* [59] demonstrated a direct coupling of CDMPC to an epoxy-activated silica-based monolith through a ring opening reaction between the epoxy groups and the hydroxy groups of the chiral selector (**Fig. 11b**). A high Rs value of 6.20 was reached for racemic 2,2'-dihydroxy-6,6'-dimethylbiphenyl on this CDMPC-functionalized column. A few reports also mentioned the use of direct post-modification for introducing chiral selectors (such as β-CD and its derivatives [9, 41, 45] and proteins [74, 184]) onto the polymer surface. Tian *et al.* [45] prepared a β-CD-functionalized monolith *via* a direct ring opening reaction between the amine groups of 4-dimethylamino-1,8-naphthalimide-β-CD (DMAN-β-CD) and the epoxy groups of the poly(GMA-*co*-EDMA) monolith (**Fig. 11a**). HSA [74] and pepsin [184] -functionalized monoliths were also fabricated through similar reactions. However, these polymer-based columns exhibited quite low enantioresolving power, like for example, in the case of warfarin on the HSA-functionalized monolith (*Rs*=0.36) [74]. This could be attributed to an insufficient content of the immobilized chiral selector in the monolith [72, 74, 75], which may become an even more severe issue in the case of large molecular weight chiral selector

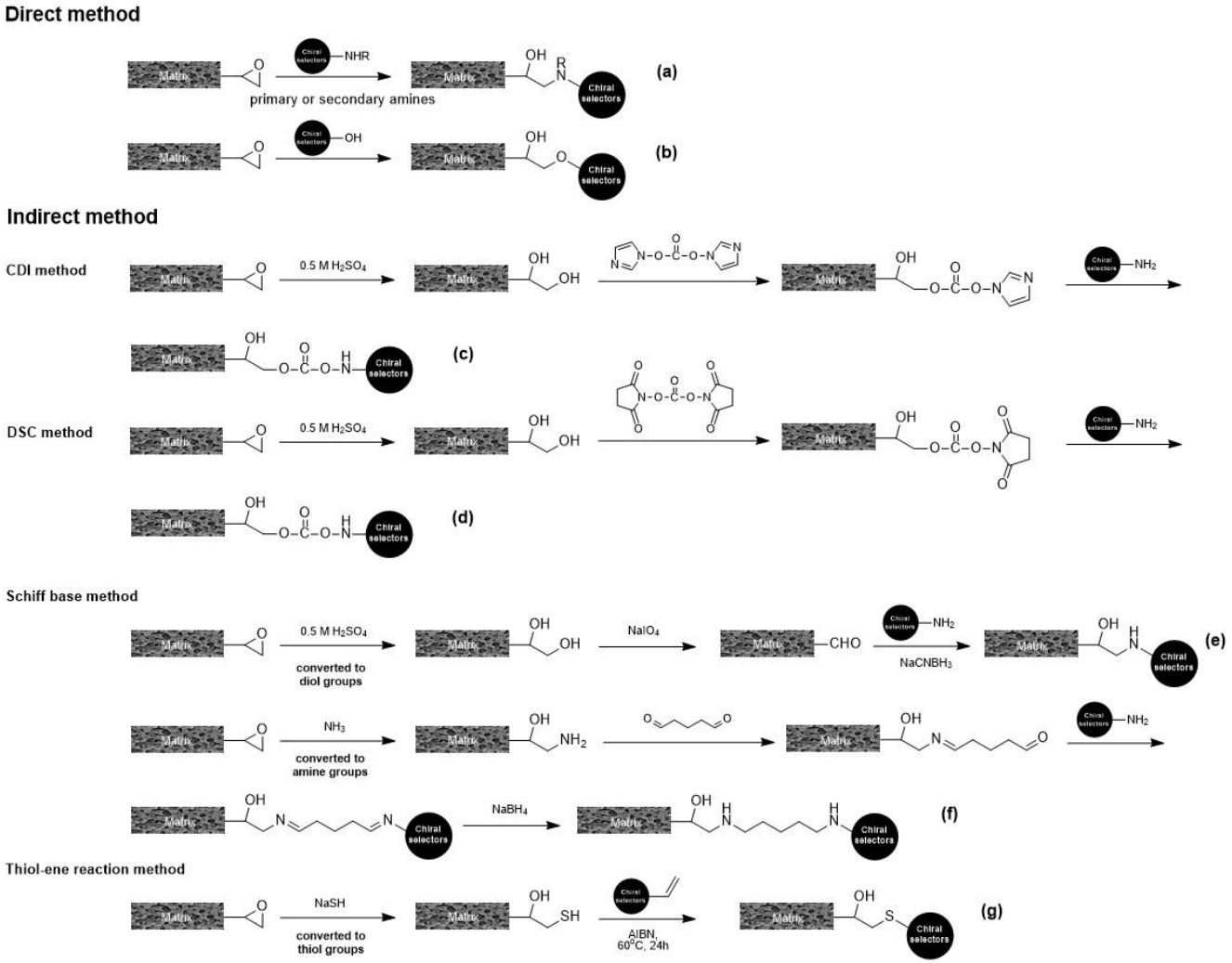


Fig. 11a-11g. Direct and indirect methods used for the immobilization of chiral selectors to the epoxy-activated monolithic matrix

In order to increase the amount of chiral selector in the monolithic polymer, additional steps are required to first convert the epoxy groups to diol, amino or thiol groups [124], then introduce other more reactive functionalities (such as aldehyde [76], imidazolyl carbamate [74] or succinimide groups [74]), and finally graft the chiral selector. **Fig. 11** shows several typical indirect post-modification methods for immobilizing chiral selectors onto an epoxy-activated monolithic matrix, such as carbonyldiimidazole (CDI), disuccinimidyl carbonate (DSC), Schiff base and thiol-ene reaction methods. Briefly, the CDI method (**Fig. 11c**) involves the hydrolysis of the epoxy groups to yield diol groups, nucleophilic substitution reaction with CDI to produce imidazolyl carbamate groups, and a further reaction with the amino groups of the chiral selector to form a stable amide linkage. The DSC method (**Fig. 11d**) is similar to the CDI method. The diol groups react with DSC to place succinimidyl carbonate groups on the surface, which then react with the primary amino groups of the chiral selector to form a carbamate linkage. In the Schiff base method, the epoxy groups are first converted to diol groups (**Fig. 11e**) or amino groups (**Fig. 11f**), which are further modified to aldehyde groups, which then react with the primary amino groups of the chiral selector to form a Schiff base. Kornysova *et al.* [93] successfully employed the Schiff base method (**Fig. 11e**) to couple vancomycin to a poly(AGE) monolith. In the thiol-ene method (**Fig. 11g**), the epoxy groups are first converted to thiol groups [124], which then react with the chiral selector containing a double bond. This method will be discussed further below.

Several studies have compared the monolithic CSPs prepared through direct or indirect post-modification methods. For instance, Hage's group [74] compared the efficiency of one direct and three indirect methods for immobilizing HSA on the poly(GMA-*co*-EDMA) monolith. The epoxy groups of the poly(GMA-*co*-EDMA) monolith were first converted to diols, and then used to immobilize HSA through CDI, DSC or Schiff base methods, respectively. The bicinchoninic acid (BCA) protein assay results showed that the highest HSA density was obtained on the column prepared via the Schiff base method, and the resulting column showed the highest enantioresolution for warfarin and tryptophan. Interestingly, some controversial results were reported by Li's group [9] and Yao's group [76], when they tried to compare direct and Schiff base methods for immobilizing NH_2-β-CD [9] or HSA [76] on the poly(GMA-*co*-EDMA) monolith. In both cases, the Schiff base method did not show any significant advantage in terms of column enantioseparation performance.

As mentioned by Hage's group [74], succinimide groups can readily react with primary amines, thiols, as well as to lysine residues in proteins [186], through aminolysis [40], hydrolysis [187] or thiolysis [188] reactions. Therefore, the succinimide-containing reagents (e.g. NAS [40, 187, 189, 190], well-known for its reactivity towards proteins [191], and mercaptopropyltrimethoxysilane [137]) are also useful in the post-modification approach. Some chiral selectors, such as proteins [74, 137], cellulose derivatives [187] and β-CD derivatives [40] have been reported to be coupled to the succinmide-activated monoliths via direct methods. For example, Wang *et al.* [187] demonstrated an effective and simple direct coupling of cellulose *tris*(4-methylbenzoate) (CTMB) on the surface of the poly(NAS-*co*-EDMA) monolith. The baseline enantioseparation of five analytes (phenylalanine, tyrosine, tryptophan, propranolol and phenylethanol) was achieved within 1.2 min. Carbonnier *et al.* [40] reported an indirect post-modification process for introducing β-CD onto the poly (NAS-*co*-EDMA) monolith. The succinimide groups were first modified with propargylamine to introduce alkyne groups, and the latter then reacted with mono-6-azido-6-deoxy-β-CD *via* alkyne-azide reaction.

If the monoliths bear alkyne (or azide) or thiol (or vinyl) groups, click reactions, including alkyne-azide and thiol-ene reactions, might be the best choice for introducing chiral selectors to the pre-activated monolithic matrix [192]. The alkyne-azide reaction between organic azide and a terminal alkyne is the most popular click reaction used in the post-modification step [40, 43, 144]. Salwinski *et al.* [43] first prepared an alkyne-functionalized monolith, i.e. poly (propargyl acrylate-pentaerythritol triacrylate-trimethylolpropane trimethacrylate) (poly(PA-PETRA-TRIM)). Through the alkyne-azide reaction, two kinds of selectors, i.e. cinnamyl azide and β-CD azide (mono-6-azido-6-deoxy-β-CD), were directly attached. Svec *et al.* [144] prepared an azide-activated silica-based monolith by modifying monolithic silica with 3-(azidopropyl)trimethoxysilane, which then reacted with the alkynes of the proline-derived chiral selector. The resulting brush-type silica-based monolithic CSPs demonstrated good enantioselectivity for four analytes with the highest Rs value of 11.4.

Through the thiol-ene reaction, Lämmerhofer *et al.* [137] immobilized an allyl chiral selector, (*S*)-*N*-(4-allyloxy-3,5-dichlorobenzoyl)-2-amino-3,3-dimethylbutane phosphonic acid, on a thiol-activated silica-based monolith. The thiol groups were introduced by modifying the silica monolith with 3-mercaptopropyl trimethoxysilane. The same group also used the thiol-ene reaction to immobilize *t*-BuCQN on the

poly(GMA-*co*-EDMA) monolith [124]. The epoxy groups of the poly(GMA-*co*-EDMA) monolith were first converted to thiols by reaction with sodium hydrogen sulfide (NaSH). In order to increase the amount of thiol groups in the polymer matrix, Lämmerhofer *et al.* developed two other methods [120]. One method consists of replacing NaSH by poly-3-mercaptopropyl methylsiloxane (PMPMS). The other one includes three additional steps, 1) conversion of epoxy groups to amino groups (amination), 2) reaction with AGE to introduce vinyl groups (vinylation), and 3) thiol-ene click reaction with PMPMS to introduce thiols (thiolation). The latter method gave higher thiol coverage and selector density, and therefore higher enantioselectivity (α= 1.7) for 3,5-dinitrobenzoyl-leucine.

Moreover, radical polymerization was also an alternative way for post-modification [6, 18]. Yuan *et al.* [18] used 3-(methacryloxy)propyltriethoxysilane (γ-MAPS) as a bifunctional reagent to prepare a silica-based monolith with double bonds, which could copolymerize with allyl-β-CD. Finally, the immobilized β-CD on the surface was sulfated in situ with chlorosulfonic acid-pyridine, which results in a sulfated β-CD-functionalized monolith.

A benefit from independently controlling the column morphology, is that the morphology and permeability are not major concerns for the post-modification approach. After carefully optimizing the morphology of the matrix column, we can focus on the manipulation of enantioselecivity. However, how to immobilize sufficient amounts of chiral selectors in a proper coupling way currently remains a main challenge [183]. In addition, the post-modification approach is still time-consuming with unsatisfactory reproducibility [36]. More effort is required to cope with these challenges.

I.4.2. Single-step approah

In order to avoid time-consuming post-modification steps, a facile and effective "single-step" approach gained popularity in recent years (**Fig. 12**). Using commercially available or lab-synthesized chiral precursors (monomers or silico alkoxides), monolithic CSPs can be easily fabricated through an *in-situ* copolymerization or sol-gel process without further modification. For the organic polymer-based or organic-silica hybrid monolithic CSPs, the desired chiral selectors need to be converted into polymerizable chiral monomers, such as methacrylated [42], acrylated [176] or allylated [8] chiral selectors, while chiral silico alkoxides are required for preparing silica-based monolithic CSPs [96, 162]. Several review articles have described the

single-step preparation approach for monolithic CSPs [36, 47]. This approach was presented as a way to greatly simplify the preparation process [36, 47, 72].

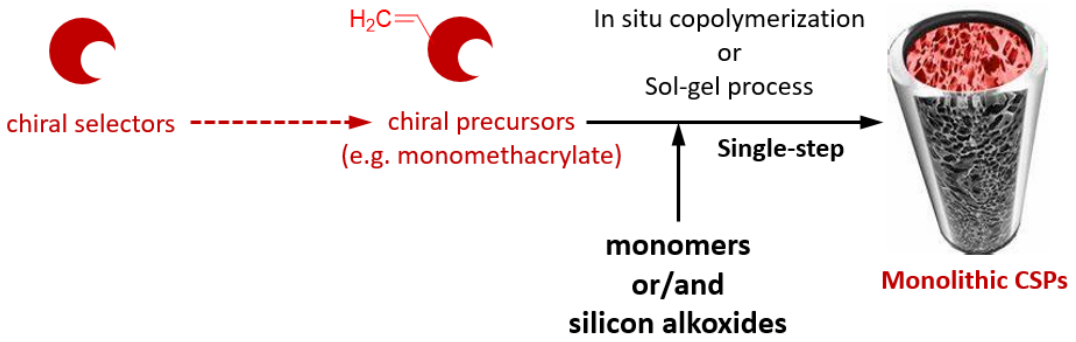


Fig.12. Scheme for the preparation of monolithic CSP *via* single-step approach.

Methacrylate or acrylate-based polymers are the most popular support for organic polymer-based monolithic CSPs. Various methacrylate and acrylate chiral monomers, such as quinidine derivatives (e.g. *t*-BuCQN) [193], vancomycin derivatives (e.g. 2-isocyanatoethyl methacrylate vancomycin (ICNEML vancomycin) [85]), amino acid derivatives [176] and β-CD derivatives [42, 48, 194], have been successfully synthesized and used for the single-step preparation of the corresponding monolithic CSPs. To date, several methacrylate/acrylate reagents, such as methacryloyl/acryloyl chloride [6, 46, 176], GMA [195, 196], propargyl methacrylate (PMA) [42, 48], and ICNEML [11, 168] have been frequently used to synthesize the methacrylated/acrylated chiral monomers.

Methacryloyl chloride is a very common reagent for methacrylation. Ghanem's group [46] synthesized the polymerizable 2,3,6-tris(phenylcarbamoyl)-β-CD-6-methacrylate through the reaction between β-CD, methacryloyl chloride and phenyl isocyanate. Acryloyl chloride was also used to synthesize three acrylamido alkenoxy carbonyl-L-leucinate (SAACL) monomers [176]. The ring opening reaction of GMA was also employed for synthesizing methacrylated chiral monomers when the chiral selector contained hydroxy groups or aminogroups, like for example glycidyl methacrylate bonded β-CD (GMA-β-CD) [195, 196]. The alkyne-azide reaction of PMA is a highly selective way to synthesize chiral monomers. Jiang and Guo [42] synthesized methacrylated β-CD monomers (propargyl methacrylate-β-CD, PMA-β-CD) through the click reaction between PMA and mono-6-azido-6-deoxy-β-CD under microwave conditions, and then copolymerized it with EDMA to produce a native β-

CD-functionalized monolithic column. To prepare other derivatived β-CD-functionalized monoliths, they further modified the PMA-β-CD monomer to PMA-methylated-β-CD and PMA-sulfated-β-CD [48]. ICNEML is also a useful reagent for methacrylation of, for example, quinidine [11] and vancomycin [168].

It is worth mentioning that the pre-synthesis of chiral monomers allows us to freely change the type of chiral selector, the position of substituents, the degree of substitution (DS) and the length of the alkyl chain linking to methacrylate/acrylate groups, which provides the possibility to study the effect of these changes on the enantioseparation performance. For example, Shamsi and Gu [195, 196] synthesized a series of GMA-β-CD monomers with different DS. It was found that the DS of GMA-β-CD has a clear effect on the enantioselectivity and column efficiency of the obtained monolithic CSPs [194, 195]. A DS of 2.0 gave the best enantioseparation performance, and a total of 32 chiral compounds could be baseline or partially enantioresolved using the proposed GMA-β-CD-functionalized monolithic column. In another research, Shamsi *et al.* [176] studied the effect of the length of the alkyl chain linked to acrylate groups on enantioseparation. Three acrylated chiral monomers, i.e. acrylamido alkenoxy carbonyl-L-leucinate (SAACL) with different chain lengths (8, 10, and 12 carbon alkyl chains) were first synthesized using acryloylamide tail, carbamate linker and L-leucine. The three corresponding leucinate-functionalized monoliths showed different enantioresolution for pseudoephedrine. The highest enantioresolution was obtained on the column containing the acrylated monomer with 12 carbon alkyl chain. Similar researches were also carried out by Jiang and coworkers for β-CD-functionalized monoliths [194].

Allylated chiral selectors were also applied to the single-step preparation of monolithic CSPs [8, 136, 197, 198]. Commonly used allylation reagents include allyl glycidyl ether (AGE), allyl bromide (AB), allyl amine (AA), etc. Chiral selectors with hydroxy or amino groups, such as β-CD and its derivatives [197, 198], could easily react with these three allylation reagents. For example, Li *et al.* [197, 198] synthesized two allyl-β-CDs, i.e. 6-(3-acryloxy-2-hydroxy)-β-CD and 2,3,6-allyl-β-CD, through reactions of β-CD with AGE or AB, respectively, and then copolymerized them *in situ* with GMA and EDMA. Some chiral selectors containing vinyl groups, such as cinchonidine [136] could be directly used as chiral monomers. For example, phenylisocyanate cinchonidin (PCD), was indirectly co-polymerized with oktakis(3-mercapto-propyl)-octasilisesquioxane (POSS-SH) and two different crosslinkers ((+)-

N,N'-diallyl-*L*-tartardiamide (DATDA) or 1,2,4-trivinylcyclohexane (TVCH)), respectively, for preparing cinchonidin-functionalized monoliths.

For silica-based monolithic CSPs, the pre-synthesis of chiral silicon alkoxides was also necessary prior to the single step sol-gel process. Hsieh *et al.* [162]first synthesized two kinds of β-CD silicon alkoxides *via* a reaction between mono-6-O-(*p*-toluenesulfonyl)-β-CD (mono-6-Ts-β-CD) and 3-aminopropyltriethoxysilane (APTES). The triethoxysilyl group was covalently attached to β-CD through secondary amine linkages. The copolymerization of β-CD silicon alkoxide with TMOS under an acid-catalyzed sol-gel process yielded a β-CD-functionalized monolithic column. Later, the same group [96] also synthesized a vancomycin silicon alkoxide *via* an addition reaction by covalently attaching triethoxysilyl group (3-isocyanatopropyltriethoxysilane (ICNPTES)) to the vancomycin derivative. A vancomycin-functionalized monolithic column was then produced through the copolymerization of the vancomycin silicon alkoxide with TMOS under an acid-catalyzed sol-gel process. More recently, Park's group [103] synthesized a carbamoylated azithromycin (also for clindamycin phosphate and erythromycin) as co-precursor via the reaction with 3-triethoxysilylpropylisocyanate, and then prepared the corresponding zirconia hybrid monolith.

The single-step approach combines the control of monolithic morphology and the manipulation of enantioselectivity. However, a personalized optimization of the copolymerization conditions is unavoidable in each case in order to gain both satisfactory morphology and enantioselectivity. Moreover, the desired chiral precursors are not always commercially available, and their synthesis is sometimes difficult. Besides, the question of whether or not the chiral selectors will be encapsulated inside the monolithic skeleton and then affect enantioselectivity is still a controversial issue for the single-step approach.

I.4.4. One-pot copolymerization approah

A one-pot synthesis is a strategy to improve the efficiency of a chemical reaction whereby a reactant is subjected to successive chemical reactions in a single reaction vessel [199]. This approach intends to avoid lengthy purification procedures of the intermediates. The term of "one-pot" has been used by several groups (i.e. Ghanem's, Jiang's and Zou's groups) for describing their strategies for preparing monolithic CSPs [8, 44, 82]. Ghanem *et al.* used the "one-pot" expression to describe their approaches

for preparing trimethylated-β-CD (TM-β-CD) [174] or a lipase [82]-functionalized organic polymer-based monolithic capillary column. In both cases, the chiral selectors (TM-β-CD or 6% lipase aqueous solution in potassium phosphate buffer) were directly added into the copolymerization mixture. The chiral selectors were encapsulated into the monolithic matrix simultaneously during the single-step copolymerization. In the strict sense of the term, it is not a real one-pot synthesis since there is no successive chemical reactions in the same pot.

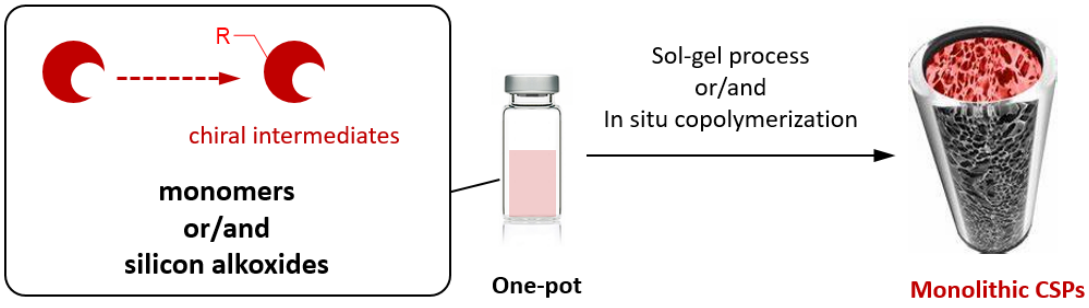


Fig. 13. Scheme for the preparation of monolithic CSP *via* the one-pot approach.

Jiang and Guo developed a one-pot approach, which combines the synthesis of a chiral monomer and subsequent copolymerization in the same vial, for preparing CD-functionalized organic polymer-based monoliths (**Fig. 13**) [44, 194, 200]. The ring opening reaction between the epoxy groups of GMA and the primary amines of ethylenediamine-β-CD (EDA-β-CD) yielded a polymerizable chiral monomer, i.e. glycidyl methacrylate-ethylenediamine-β-CD (GMA-EDA-β-CD), through the sequential copolymerization step without purification. Practically, it further simplified the single-step copolymerization procedure. They also studied the influence of the crosslinker type, linking spacer length and type on the enantioseparation ability of the monolithic CSPs. Compared to the previously reported post-modification (two-step) approach [41], the monolith prepared *via* the one-pot approach exhibited higher ligand density, satisfactory permeability and enantioselectivity (the highest Rs value was 5.07 for 2-chloro-mandelic acid).

Subsequently, this one-pot approach was applied to CDs without an amine group. Deng and Guo *et al.* [201, 202] used a basic catalyst diazabicyclo [5.4.0] undec-7-ene (DBU) to speed up the ring opening reaction between native β-CD (or HP-β-CD [201]) and GMA, and the subsequent copolymerization in one pot. Six basic drugs were

baseline enantioseparated, with the highest Rs value being 4.70 for homatropine [202]. Putri *et al.* [203] also prepared a poly (GMA-glucuronyl glucosyl β-CD (GUG-β-CD)-*co*-EDMA) monolith through the one-pot strategy, but no catalyst was employed for the ring opening reaction between GMA and GUG-β-CD.

Zou *et al.* [204] described another type of one-pot approach for preparing achiral and chiral organic-silica hybrid monolithic columns. The sol-gel process and the copolymerization of the organic and inorganic monomers were successively carried out in the same capillary. In their earlier study, two different achiral monomers, i.e. the hydrophobic monomer allyldimethyldodecylammonium bromide (ADDAB) and the hydrophilic monomer acrylamide (AA) were mixed with hydrolyzed TMOS and vinyltrimethoxysilane (VTMS) respectively, and the resulting homogeneous mixtures were introduced into fused capillaries for the subsequent polycondensation and copolymerization to form the hybrid monolithic columns. Later on, this one-pot method was applied to prepare a Ph-β-CD-functionalized hybrid monolith. It is worth pointing out that the chiral monomer mono (6-*N*-allylamino-6-deoxy)-perphenylcarbamoylated-β-CD (Ph-β-CD) needs to be pre-synthesized, starting from the readily available Ts-β-CD, followed by allylation with AA and phenylation with phenyl isocyanate.

Although the one-pot approach is simple and effective, it has only been applied in very few cases because of some inherent restrictions. For example, the byproducts generated in one reaction may interfere with the subsequent reaction, and thus it is important to choose and optimize the reactions in order to minimize the generation of undesired side-products. Besides, the solvent system empolyed in one reaction may not be suitable for another one. The selecting of suitable reaction solvents for all successive reactions is also a key point for the preparation of monolithic CSPs by the one-pot approach.

I.5. Conclusions and future perspectives

As mentioned above, numerous monolithic CSPs have been developed and applied to the enantioseparation of a variety of chiral analytes, including amino acids, small peptides, basic and acidic drugs, such as α- and β-blockers, NSAIDs, barbituric acid derivatives, antifungal drugs, dopamine antagonists, sedative hypnotics, antihistamines, etc. These monolithic CSPs displayed some desirable features, such as low flow-resistance, fast enantioseparation, and good enantioselectivity.

However, several major challenges need to be overcome before bringing even one

monolithic chiral column to the market. One main challenge is how to prepare monolithic CSPs with good reproducibility in terms of morphology, permeability, enantioselectivity and column efficiency. This is a common problem existing with monolith preparation. So far, most monolithic CSPs are still prepared *in situ* inside a chromatographic column, which is not an ideal reactor for uniform reaction and process monitoring. Besides, both sol-gel processes and copolymerization are quite sensitive to reaction conditions. Minor variations in the reaction mixture composition and temperature (or UV intensity) may lead to significant differences in morphology and final separation performance. Secondly, most of the reported monolithic CSPs are small size capillary columns, which largely limits a wider application. It will be of great interest to prepare monolithic CSPs possessing high column efficiency, good mechanical strength and good enantioselectivity in large-scale columns. Thirdly, a few monolithic CSPs have been applied to the analysis of real samples. This could be attributed in many cases to their lower enantioresolution and column efficiency in comparison with their commercial particulate counterparts. The amount of chiral selector in a monolithic matrix is still to be increased, particularly for high-molecular weight chiral selectors. Moreover, it was found that the way of coating, encapsulating or covalently bonding and the chemistry for attaching chiral selectors could significantly affect their enantiorecognition. However, a comprehensive and in-depth theoretical study is still absent. The successful commercialization of achiral monoliths enables to thing that studies on the preparation approach of monolithic CSPs will still be the mainstream for some time.

The information included in this section is extended in a review article prepared in the context of this PhD Thesis：

Rewiew article: *Recent advances in preparation and applications of monolithic chiral stationary phases.*

J. Guo, Q. Wang, D. Xu, J. Crommen, Z. Jiang.

Trends Anal. Chem.*, submitted.*

CHAPTER II
OBJECTIVES

Many possibilities are nowadays available to carry out a successful chiral separation, in which nano-separative techniques play a vital role due to the advantages derived from their inherent low dimensions. Among the different miniaturized strategies to achieve chiral separations, the use of chiral monolithic columns in nano-liquid chromatography (nano-LC) or capillary electrochromatography (CEC) has attracted much interest due to their high column efficiency, increased sensitivity and the ease of coupling to Mass Spectrometry as well as reduced solvent and sample consumption.

Although numerous chiral stationary phases (CSPs) have been reported, based on cyclodextrins and their derivatives, quinidine and its derivatives, cellulose derivatives, proteins, macrocyclic antibiotics, crown ethers and chiral ion-exchangers, the development of novel CSPs presents a high interest. In the last years, many efforts have been focused on the development of organic polymer-based or organic-inorganic hybrid-based chiral monolithic columns due to their excellent permeability, pH stability, high performance and simple preparation.

So far, very few works reported the preparation of functionalized polymer-based or organic-inorganic hybrid-based chiral monoliths based on macrocyclic antibiotics and their derivatives. This could be due to some disadvantages associated with the multi-step preparation strategy that are related to its complexity and unsatisfactory repeatability, and to the fact that it is time-consuming. In order to reduce the time and work necessary to prepare macrocyclic antibiotics functionalized organic polymer-based or organic-silica hybrid monoliths, the development of a single-step approach would be very interesting. With this purpose, vancomycin and teicoplanin functionalized organic polymer-based and organic-silica hybrid monoliths were developed in this PhD Thesis by a single-step.

Moreover, quinidine functionalized organic-silica hybrid monoliths have never been reported before, only a few have been reported so far being based on the so called multistep approach for quinine functionalized hybrid monoliths. Taking this into account, it would seem appropriate to adapt the synthesis of quinidine functionalized hybrid monolith as a “one-pot” and this was one of the objectives of this PhD Thesis. In addition, in the previous works dealing with quinidine functionalized monoliths, few works reported applications. Therefore, a method was developed in this PhD Thesis enabling the determination of the content of L-norvaline and L-tryptophan in dietary supplements based on the use of the quinidine functionalized organic-inorganic hybrid-based monoliths developed in this work by nano-LC.

To achieve these goals, the following specific objectives were proposed:

Article 1.

- To synthesize the vancomycin functional chiral monomer using ICNEML.
- To prepare a vancomycin functionalized polymer-based monolithic column and to optimize the most adequate polymerization conditions.
- To optimize the chromatographic conditions for enantioseparation.
- To evaluate the potential of the developed poly(ICNEML-vancomycin-*co*-EDMA) monolithic column for drug enantioseparation.

Article 2.

- To synthesize the teicoplanin functional chiral monomer using ICNEML.
- To develop a single-step approach to prepare a teicoplain organic-silica hybrid monolithic column and to optimize the preparation process.
- To optimize the chromagotraphic conditions for the enantioseparation of amino alcohols, *N*-derivatized amino acids and mandelic acids.
- To investigate the influence of the matrix by comparing with a polymer based poly(ICNEML-vancomycin-*co*-EDMA) monolithic column.

Article 3.

- To prepare a MQD functionalized organic-silica hybrid monolithic column by a facile "one-pot" approach.
- To evaluate the MQD organic-silica hybrid monolithic column for amino acid enantioseparation under polar-organic and reversed-phase modes.
- To compare with poly(MQD-*co*-HEMA-*co*-EDMA) monolithic column regarding efficiency, enantioresolution, analysis time, and enantioselectivity.

Article 4.

- To select the most adequate derivatization reagent for amino acid separation in the MQD functionalized organic-silica hybrid monolithic column.
- To optimize the enantiomeric separation of amino acids.
- To apply the developed method to the enantiomeric determination of L-Norvaline and L-Tryptophan in dietary supplements.

CHAPTER III
RESULTS

III. 1.

Preparation of vancomycin functionalized polymer-based monoliths as chiral stationary phase for nano-liquid chromatography

III.1. Preparation of vancomycin functionalized polymer-based monoliths as chiral stationary phase for nano-liquid chromatography

III.1.1. Preface

As mentioned in the introduction section of this work, although numerous chiral stationary phases (CSPs) are commercially available, the development of novel CSPs presents a high interest. Recently, increasing efforts have been directed toward the development of organic polymer-based chiral monolithic columns because of their excellent permeability, pH stability, low resistance to mass transfer and high performance. So far, various chiral functionalized polymer-based chiral monoliths have been reported.

Vancomycin-type glycopeptide antibiotics have been proved to be a versatile class of chiral selectors for enantioseparation in polar organic-phase, normal-phase and reversed-phase modes due to their enantioselectivity. However, very few vancomycin functionalized polymer-based monoliths have been reported. So far, only Maruška and co-workers developed a multi-step post-column modification strategy for immobilizing vancomycin onto the surface of organic polymeric monolith at the turn of the century. The polymeric support was prepared through *in situ* co-polymerization of N-(hydroxymethyl) acrylamide, allyl glycidyl ether and piperazine diacrylamide with vinyl sulfonic acid within 100 μm I.D. capillaries. Subsequently, vancomycin was introduced onto the polymeric skeleton *via* reductive amination of the aldehyde groups converted from epoxy groups. Later on, they simplified the preparation procedure by replacing allyl glycidyl ether with *N,N'*-diallyltartardiamide, which can be easily cleaved into two aldehyde groups using periodate treatment. The vancomycin functionalized polymer-based monoliths prepared through both ways exhibited good enantioselectivity for racemic compounds in CEC. However, there is no other report about vancomycin functionalized polymer-based monoliths. This may be partially attributed to some disadvantages associated with multi-step preparation strategy, such as be time-consuming, laborious, and probably unsatisfactory repeatability. Vancomycin functionalized silica-based monoliths were also prepared through multi-step preparation strategy. Hsieh *et al.* recently developed a single-step *in situ* sol-gel approach for preparing vancomycin functionalized silica-based monolith. A sol-gel precursor containing vancomycin was synthesized and co-polymerized with skeleton precursor to form a porous silica-based monolith. The proposed single-step approach

not only resulted in a chiral column with good efficiency and enantioselectivity for many basic enantiomers, but also significantly simplified the preparation procedure. Aiming at reducing the time and work associated to the preparation of vancomycin functionalized organic polymer-based monoliths, it would be of high interest to develop a single-step co-polymerization approach as well.

III.1.2. Objectives

The specific objectives of this chapter were:

- To prepare the vancomycin functional monomer for the monolithic column.
- To optimize the synthesis methods to increase yield and characterize the products.
- To prepare novel vancomycin functionalized polymeric capillary columns by a facile strategy.
- To optimize the vancomycin functionalized polymeric capillary column to enchance the enantioresolution, enantioselectivity and column efficiency.
- To evaluate and characterize the chromatographic performance of the novel monolithic columns.
- To investigate their chiral recognition ability for different enantiomers in different separation modes.

III.1.3. Results

The results obtained in this work are included in the following scientific article:

Article 1: *A facile and efficient single-step approach for the fabrication of vancomycin functionalized polymer-based monolith as chiral stationary phase for nano-liquid chromatography.*

D. Xu, H. Shao, R. Luo, Q. Wang, E. Sánchez-López, S. Fanali, M. L. Marina, Z. Jiang. *J. Chromatogr. A, 2018, 1557, 43-50.*

Article 1

A facile and efficient single-step approach for the fabrication of vancomycin functionalized polymer-based monolith as chiral stationary phase for nano-liquid chromatography

D. Xu, H. Shao, R. Luo, Q. Wang, E. Sánchez-López, S. Fanali, M. L. Marina, Z. Jiang

J. Chromatogr. A, 2018, 1557, 43-50.

Abstract

A facile single-step preparation strategy for fabricating vancomycin functionalized organic polymer-based monolith within 100 μm fused-silica capillary was developed. The synthetic chiral functional monomer, i.e 2-isocyanatoethyl methacrylate (ICNEML) derivative of vancomycin, was co-polymerized with the cross-linker ethylene dimethacrylate (EDMA) in the presence of methanol and dimethyl sulfoxide as the selected porogens. The co-polymerization conditions were systematically optimized in order to obtain satisfactory column performance. Adequate permeability, stability and column morphology were observed for the optimized poly(ICNEML-vancomycin-*co*-EDMA) monolith. A series of chiral drugs were evaluated on the monolith in either polar organic-phase or reversed-phase modes. After the optimization of separation conditions, baseline or partial enantioseparation were obtained for series of drugs including thalidomide, colchicine, carteolol, salbutamol, clenbuterol and several other β-blockers. The proposed single-step approach not only resulted in a vancomycin functionalized organic polymer-based monolith with acceptable performance, but also significantly simplified the preparation procedure by reducing time and labor.

Keywords:

Vancomycin, Enantioseparation, Organic polymeric monolith, Nano-LC

1. Introduction

Although a large number of chiral stationary phases (CSPs) are available on the market, the development of novel CSPs still attracts considerable interest [1-2]. Recently, increasing efforts have been directed toward the development of organic polymer-based chiral monolithic columns because of their excellent permeability, pH stability, low resistance to mass transfer and high performance [3-5]. So far, various chiral functionalized polymer-based chiral monoliths have been reported, such as cyclodextrin and its derivatives [6-7], quinidine and its derivatives [8-10], cellulose derivatives [11], proteins [12, 13], macrocyclic antibiotics [14, 15], crown ethers [16] and chiral ion-exchangers [17].

Over the years, the vancomycin-type glycopeptide antibiotics have been proved to be a versatile class of chiral selectors for enantioseparation in polar organic-phase, normal-phase and reversed-phase modes since their enantioselectivity was demonstrated by Armstrong *et al.* [18]. However, very few vancomycin functionalized polymer-based monoliths have been reported. So far, only Maruška and co-workers developed a multi-step post-column modification strategy for immobilizing vancomycin onto the surface of organic polymeric monolith at the turn of the century [14, 15]. The polymeric support was prepared through in situ co-polymerization of N-(hydroxymethyl) acrylamide, allyl glycidyl ether and piperazine diacrylamide with vinyl sulfonic acid within 100 μm I.D. capillaries. Subsequently, vancomycin was introduced onto the polymeric skeleton via reductive amination of the aldehyde groups converted from epoxy groups. Later on, they simplified the preparation procedure by replacing allyl glycidyl ether with N, N'-diallyltartardiamide, which can be easily cleaved into two aldehyde groups using periodate treatment. The vancomycin functionalized polymer-based monoliths prepared through both ways exhibited good enantioselectivity for racemic compounds in capillary electrochromatography (CEC). However, the authors did not provide any column-to-column and batch-to-batch repeatability data in their studies. To the best of our knowledge, there is no other report about vancomycin functionalized polymer-based monoliths. This may be partially attributed to some disadvantages associated with multi-step preparation strategy, such as time-consuming, laborious and probably unsatisfactory repeatability. Vancomycin functionalized silica-based monoliths were also prepared through multi-step preparation strategy [19-22]. Hsieh *et al.* recently developed a single-step in situ sol-gel approach for preparing vancomycin functionalized silica-based monolith [23]. A

sol-gel precursor containing vancomycin was synthesized and co-polymerized with skeleton precursor to form a porous silica-based monolith. The proposed single-step approach not only resulted in a chiral column with good efficiency and enantioselectivity for many basic enantiomers, but also significantly simplified the preparation procedure. Aiming at reducing the time and labor associated with the fabrication of vancomycin functionalized organic polymer-based monoliths, it would be of high interest to develop a single-step co-polymerization approach as well.

In this work, a chiral functional monomer, i.e 2-isocyanatoethyl methacrylate (ICNEML) derivative of vancomycin (ICNEML-vancomycin), was first synthesized. It was then in situ co-polymerized with the cross-linker ethylene dimethacrylate (EDMA) in a binary porogen system of methanol and DMSO. The polymerization conditions were systematically optimized in order to obtain satisfactory permeability, column efficiency and enantioresolution. The enantioresolution capability of the optimized monolith was evaluated by analyzing a series of chiral drugs in either polar organic-phase or reversed-phase modes. The enantioseparation conditions, including the organic solvent type and concentration, the buffer concentration and the pH of the mobile phase, were also carefully optimized.

2. Materials and methods

2.1. Reagents and samples

2,2'-azobisisobutyronitrile (AIBN), 3-(trimethoxysilyl)-propylmethacrylate (γ-MAPS), ethylene dimethacrylate (EDMA), 2-isocyanatoethyl methacrylate (ICNEML), DMSO, methanol (MeOH), ethanol, 1,4-butanediol, 1-propanol, tetrahydrofuran (THF), cyclohexane, 1-dodecanol and toluene, acetonitrile (ACN), triethylamine (TEA), acetic acid (HAc), pyridine, acetone and vancomycin hydrochloride were acquired from Aladdin Chemicals (Shanghai, China). Acebutolol, carteolol, sotalol, propranolol, pindolol, tertaolol, clenbuterol, salbutamol and thalidomide were obtained from Energy Chemical (Shanghai, China). Colchicine was purchased from Sigma (Missouri, MO, USA). The fused-silica capillaries (375 μm O.D. × 100 μm I.D.) were obtained from Ruifeng Chromatography Ltd. (Hebei, China). Distilled water was purified using a Milli-Q system (Massachusetts, MA, USA). Polar organic mobile phases were set up by mixing the desired ratio of ACN and MeOH, and then adding various amount of TEA and HAc. Reversed-phase mobile phases were prepared by mixing the

corresponding ratio of MeOH or ACN and buffer containing TEAA. All mobile phases were subjected to filtration through a 0.22-μm membrane and sonication degas prior to use.

2.2. Instrumentation

Molecular masses were determined on a Waters Synapt G2 TOF mass spectrometer (Milford, MA, USA). A Jinghong DKS22 water bath (Shanghai, China) was used for thermally initiated co-polymerization. Scanning electron microscopy (SEM) experiments were performed with a Zeiss Gemini ultra-55 SEM (Deutschland, Germany) at an acceleration voltage of 5 kV. All nano-LC experiments were conducted on a nano-LC instrument, laboratory assembled. The system consists of a DiNa nano gradient pump (Tokyo, Japan), a Shimadzu SPD-15C UV detector (Kyoto, Japan) with a lab-made on-column detection cell and a Valco four-port injection valve with 20 nL internal loop (Houston, TX, USA). All data acquisition and analysis were carried out with Unimicro Trisep^{TM} Workstation 2003 (Shanghai, China). The pH values of buffer solutions were measured by a Sartorius PB-10 pH meter (Göttingen, Germany).

2.3. Synthesis of the chiral functional monomer ICNEML-vancomycin

The nucleophilic addition of amine or hydroxyl groups were often used for the derivatization of vancomycin [24, 25]. In this study, ICNEML was chosen as the derivatization reagent to modify vancomycin through the nucleophilic addition reaction. For the schematic representation of the synthesis of the novel ICNEML-vancomycin monomer, see **Fig. 1**. In brief, vancomycin hydrochloride (60 mg, 0.04 mmol) was dissolved in DMSO (0.3 mL). Then, pyridine (0.4 mL) and ICNEML (10 μL, 0.07 mmol) were added into the mixture and stirred for 24 h under nitrogen at room temperature. After adding acetone (8 mL) and stirring for another 10 min, a white precipitate was collected by centrifugation at 4000 rpm for 5 min and washed with acetone for five times. Finally, the precipitate was dried under vacuum to give the target monomer (light white solid). The molecular formula of ICNEML-vancomycin was established as $C_{73}H_{84}N_{10}O_{27}Cl_2$ from its HR-ESI-MS (m/z: 1603.4965 $[M+H]^+$, calculated for $C_{73}H_{85}N_{10}O_{27}Cl_2$: 1603.4963) in **Fig. S1**.

2.4. Preparation of the poly(ICNEML-vancomycin-co-EDMA) monolith columns

Prior to the polymerization, the fused-silica capillaries were pretreated with γ-MAPS

to provide the anchoring sites for the bulk polymer [26]. Then, the monomer ICNEML-vancomycin, the binary porogens (DMSO and MeOH), the crosslinker EDMA and the initiator AIBN were accurately weighted and mixed into a homogenous solution in a 2 mL of vial. The mixture was sonicated and degassed for 5 min, and then introduced into 20 cm long pre-treated capillaries. Both ends of the capillaries were sealed with rubber plugs and submerged into the water bath at 60 ºC for 12 h. The unreacted porogens and chemicals were removed by flushing the column with methanol. The obtained monolith was cut to 15 cm for nano-LC analysis. A 2-5 mm length of the monolith was used for scanning electron microscopy (SEM) analysis.

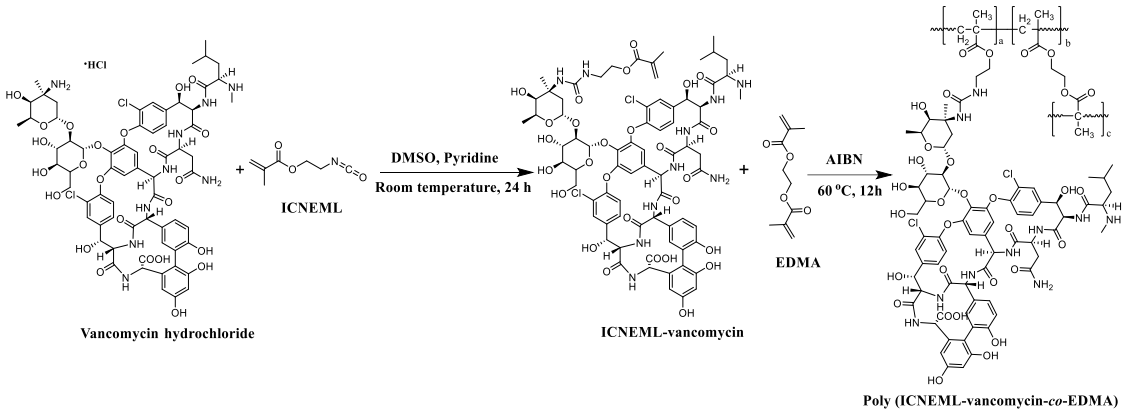


Fig. 1. Schematic representation of the synthesis of the ICNEML-vancomycin

3. Results and discussion

3.1. Preparation and characterization of the poly(ICNEML-vancomycin-co-EDMA) monolithic column

Porogen selection is a critical step in the preparation of polymer-based monolithic column since the type and amount of porogens influence the porosity, morphology, permeability and even the chromatographic efficiency of the monolith. A suitable porogenic solvent or solvent combination should be able to dissolve all components (including functional monomer, initiator and cross-linker) and does not react each other chemically. In this study, several commonly used polar solvents (DMSO, water, MeOH, ethanol, 1,4-butanediol and 1-propanol) and non-polar solvents (THF, cyclohexane, toluene, 1-dodecanol) were initially investigated. The solubility of monomers, the permeability and visual appearance of the monoliths prepared under each porogen system were inspected using nano-LC and microscopy. Based upon our initial experiments, both ICNEML-vancomycin and EDMA showed good solubility in a binary solvent system consisting of MeOH and DMSO (75/25, w/w). In addition, the

resulting monoliths also exhibited a uniform dark structure and good permeability. Therefore, these solvents were selected for the following systematical optimization of the polymerization conditions, including the weight fraction of the porogens, the weight fraction of EDMA and the composition of porogenic mixture. Acebutolol was selected as test analyte using a mobile phase consisting of MeOH/ACN/TEA/HAc (80/20/0.08/0.02, v/v/v/v). The influence of the porogen content was first studied by varying the weight fraction of MeOH/DMSO (75/25, w/w) at three different percentage, i.e. 71% (Column **C1**), 75% (Column **C2**) and 79% (Column **C3**), while keeping constant the other conditions (see **Table 1**). The results showed that the porogens content had a significant influence on the column permeability. As the percentage of porogens increased, the backpressure diminished. The column **C1** prepared with 71% porogens exhibited a very high backpressure. When comparing the enantioresolution obtained for acebutolol enantiomers, the column **C2** exhibited a higher enantioresolution, and therefore, it was selected for the following studies.

Table 1. Composition of the polymerization mixture used for the preparation of the poly (ICNEML-vancomycin-*co*-EDMA) monolith columns and their properties.

Column	Monomers (%, w/w)		Porogens (%, w/w)		Monomers: Porogens (%, w/w)		Back pressure (MPa)	Enantioresolution
	ICNEML-vancomycin	EDMA	MeOH	DMSO	Monomers	Porogens		
C1	75.0	25.0	75.0	25.0	29.0	71.0	Too high	/
C2	75.0	25.0	75.0	25.0	25.0	75.0	7.5	1.45
C3	75.0	25.0	75.0	25.0	21.0	79.0	3.4	0.37
C4	70.8	29.2	75.0	25.0	25.0	75.0	9.5	1.38
C5	79.2	20.8	75.0	25.0	25.0	75.0	3.6	0.51
C6	75.0	25.0	70.0	30.0	25.0	75.0	9.8	0.58
C7	75.0	25.0	80.0	20.0	25.0	75.0	4.7	0.94

Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: MeOH/ACN/TEA/HAc (80/20/0.08/0.02, v/v/v/v); UV detection wavelength: 230 nm; total flow rate: 400 nL/min; injection volume: 20 nL; sample: acebutolol.

Second, the content of the crosslinker EDMA in the monomer mixture was optimized since it can also influence both the column permeability and enantioselectivity. As the weight fraction of EDMA in the monomer mixture increased from 20.8% (column **C5**) to 25.0% (column **C2**), the backpressure and enantioresolution dramatically increased from 3.6 to 7.5 MPa and 0.51 to 1.45, respectively. However, further increasing the EDMA content to 29.2 % (column **C4**) resulted in a slightly lower *Rs* value (1.38) and higher backpressure (9.5 MPa) when compared to column **C2**. Thus, 25.0 % EDMA was considered for further optimizations. Finally, the influence of the porogenic mixture composition (MeOH and DMSO) was investigated by varying the weight content of MeOH from 70% (column **C6**) to 80% (column **C7**). The increase of the MeOH content caused a decrease of the backpressure from 9.8 to 4.7 MPa and an increase in the *Rs* from 0.58 to 0.94. 75 % MeOH (column **C2**) allowed for the highest *R*s value under a reasonable backpressure.

Based on these optimization experiments, the polymerization mixture containing 25% monomers (ICNEML-vancomycin/EDMA, 75/25, w/w) and 75% porogens (MeOH/DMSO, 75/25, w/w) were selected for following studies. The morphology of the optimized poly (ICNEML-vancomycin-*co*-EDMA) (column **C2**) monolithic column was evaluated by scanning electron microscopy (SEM). As shown in **Fig. 2**, the SEM images indicated that the column **C2** has a morphology of continuous skeleton and large through-pores, and the monolithic rod is tightly anchored on the inner wall of the capillary column.

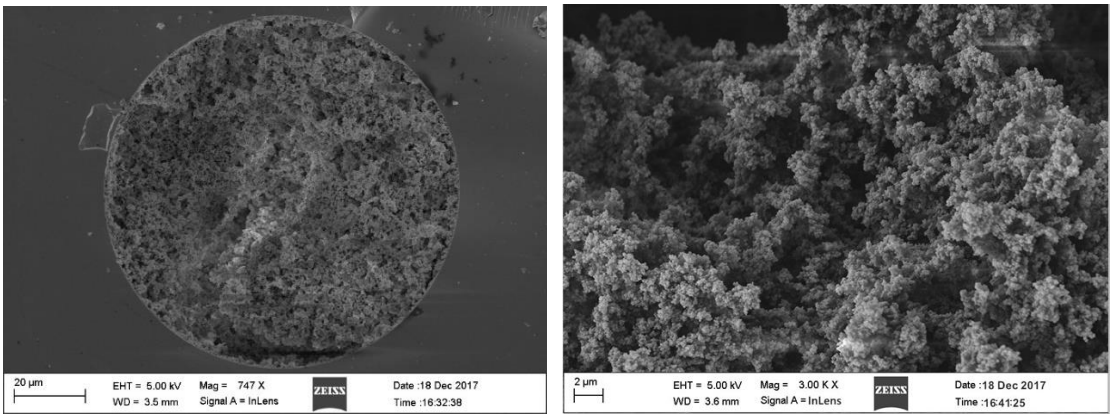


Fig. 2. SEM images of the poly (ICNEML-vancomycin-*co*-EDMA) monolithic column at different magnifications.

3.2. Permeability and reproducibility of the poly(ICNEML-vancomycin-co-EDMA) monolithic columns

The permeability K of a monolithic column can be calculated according to the following equation [27, 28]:

$$K = \frac{u\eta L}{\Delta P}$$

where u is the linear velocity of the mobile phase, L is the length of the column, ΔP is the pressure drop across the column, and η is the dynamic viscosity of the eluent. Toluene (ACN or MeOH as polar mobile phase) and thiourea (water/ACN (50/50, v/v) as reversed phase mobile phase) were chosen as the dead time markers. As shown in **Table 2**, the calculated K values for the column **C2** were 2.78×10^{-14}, 4.16×10^{-14} and 1.97×10^{-14} m^2 when using MeOH, ACN and water/ACN (50/50, v/v) as the mobile phases, respectively. It is worth noting that these determined permeability values are quite similar, indicating the swell or shrink of the optimized poly(ICNEML-vancomycin-*co*-EDMA) monolith in solvents with different polarities is little.

Table 2. Permeability of the poly (ICNEML-vancomycin-*co*-EDMA) monolith column

Mobile phase	Relative polarity	Viscosity η ($\times 10^{-3}$ Pa·s)	Permeability K ($\times 10^{-14}$ m^2)
ACN/H_2O (50/50)	/	0.820	1.97
MeOH	0.762	0.544	2.78
ACN	0.460	0.369	4.16

Relative polarity and viscosity data of pure liquids were obtained from Ref. [26-27]

Repeatability and reproducibility of some studied parameters on the poly(ICNEML-vancomycin-*co*-EDMA) monolithic column were evaluated through calculating the RSD values for k_1, k_2, α and Rs of the racemic test compound acebutolol using a mixture of MeOH/ACN/TEA/HAc (85/15/0.08/0.02, v/v/v/v) as mobile phase (**Table 3**). The column-to-column reproducibility (n=6) for retention factors (k_1 and k_2) were 2.36%, while the batch-to-batch (n=6) RSD values were 2.72% and 2.36%. The run-to-run repeatability (n=6) for k_1 and k_2 was also adequate with RSD values of 1.98% and 2.36%, in addition to day-to-day repeatability (n=6) which RSD values were 3.03% and 3.42%, respectively. RSD values of α and Rs were also satisfactory (≤ 5.92%). These data demonstrated that the poly(ICNEML-vancomycin-*co*-EDMA) monolithic column

has a satisfactory reproducibility for enantioseparation in nano-LC.

Table 3. Reproducibility of the poly (ICNEML-vancomycin-*co*-EDMA) monolith columns

	Average retention factor (RSD)		Average selectivity α (RSD)	Average resolution *Rs* (RSD)
	k_1	k_2		
Column to column (n=6)	1.31 (2.36%)	1.53 (2.36%)	1.17 (2.02%)	1.40 (5.92%)
Run to run (n=6)	1.27 (1.98%)	1.46 (2.36%)	1.15 (1.57%)	1.42 (4.14%)
Day to day (n=6)	1.25 (3.03%)	1.51 (3.42%)	1.21 (1.13%)	1.44 (4.72%)
Batch to batch (n=6)	1.29 (2.72%)	1.49 (2.36%)	1.16 (1.79%)	1.39 (5.33%)

Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: MeOH/ACN/TEA/HAc (85/15/0.08/0.02, v/v/v/v); UV detection wavelength: 230 nm; total flow rate: 400 nL/min; injection volume: 20 nL; sample: acebutolol.

3.3. Application of the poly(ICNEML-vancomycin-co-EDMA) monolithic column

3.3.1. Polar organic phase mode

Based on our experience and on the data reported in literature [29] a mobile phase containing ACN-MeOH and TEA-HAc was selected as polar organic mobile phase. It has been reported that in any LC-based enantioseparation, the composition of mobile phase affects the enantioselectivity through changing the charge-charge interaction, hydrogen bonding and π-π interaction, among other factors [30, 31]. Therefore, the MeOH/ACN content and additives content (TEA/HAc ratio and their total concentration) were modified to evaluate their effect on the enantioresolution of carteolol, acebutolol and sotalol. Due to the fact that the observed behavior was quite the same for these three compounds, only figure of merits for carteolol will be shown. The influence of the concentration ratio of MeOH/ACN on the retention factor and enantioresolution for carteolol was evaluated by keeping constant TEA/HAc content. As shown in **Fig. 3a** and **b**, with increasing MeOH concentration from 60% to 85 % (v/v), the enantioresolution increased reaching its maximum value, and then decreased when the MeOH concentration further raised from 85% to 100% (v/v). The retention factor (k_1) decreased gradually with the increase of the concentration of MeOH. On the other hand, the enantioselectivity factor (α) increased by raising MeOH content, while the column efficiency increased with the increase of MeOH content from 60% to 90%

(v/v) and then diminished at 100% (v/v). As a compromise between enantioresolution and column efficiency, 85% (v/v) MeOH was chosen as the mobile phase. These results also agreed with the previous studies on the vancomycin based chiral stationary phases [22] because higher MeOH content in combination with a small amount of acid/base additives might contribute to less nonselective hydrogen bonding interactions for carteolol enantiomers and vancomycin stationary phases.

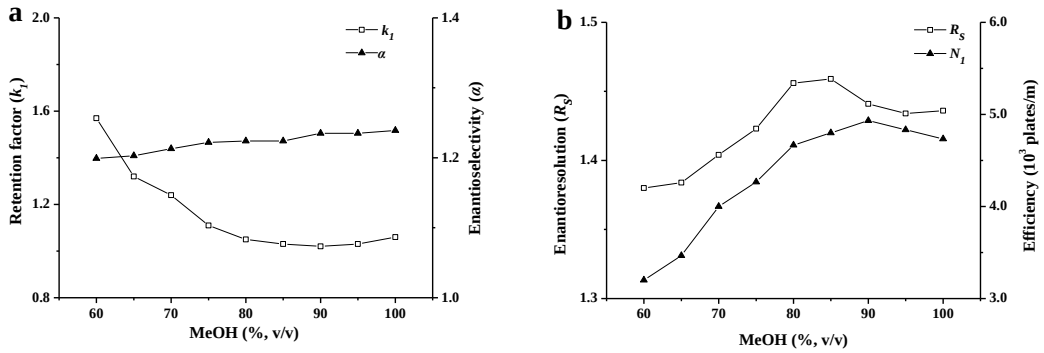


Fig. 3. Effect of the MeOH content on (a) retention factor and enantioselectivity; (b) enantioresolution and column chromatographic efficiency for carteolol enantiomers in the polar organic-phase mode. Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: MeOH/ACN/TEA/HAc (at the desired ratio of MeOH and ACN/0.08/0.02, v/v/v/v); UV detection wavelength: 230 nm; total flow rate: 400 nL/min; injection volume: 20 nL.

As can be observed in **Fig. 4a**, the use of an appropriate TEA/HAc concentration and ratio could be of paramount importance in influencing both enantioseparation and column efficiency. Therefore, the ratio of the TEA/HAc (%, v/v) in the mobile phase was varied from1:3 to 9:1, while the total concentration of TEA and HAc was kept at 0.1% (v/v) and the ratio of MeOH/ACN kept constant at (85/15, v/v). The increase of the ratio of TEA/HAc from 1:3 to 4:1 caused an increase of enantioresolution. Then this parameter decreased as the ratio further increased to 9:1. This is because the hydrogen bonding is the most important in this mode, so the stronger interaction between the CSP and enantiomer with the Ac^- content decreased in the mobile phase. However, the ionization of the basic compounds was weak as the TEA/HAc ratio increased from 4:1 to 9:1, and this would weaken the interaction. On the contrary, the chromatographic efficiency showed a different trend (decreased almost linearly by increasing the TEA/HAc ratio from 1:3 to 9:1). As a compromise to achieving optimum enantioresolution and column chromatographic efficiency, the ratio of 4:1 for TEA/HAc (0.08%/0.02%, v/v) was chosen as the mobile phase additive.

Fig. 4b shows the effect of total concentration of TEA and HAc on the column efficiency. As can be seen, the increase of the total concentration of TEA and HAc in the mobile phase from 0.01% to 0.2% (v/v) caused a raising of number of theoretical plates, while the highest enantioresolution was obtained at 0.1 % (v/v). Therefore, a total concentration of TEA and HAc of 0.1% (v/v) was selected as the optimum mobile phase modifier mixture.

Under the optimal conditions (mobile phase consisting of MeOH/ACN/TEA/HAc (85/15/0.08/0.02, v/v/v/v)), eight racemic compounds were tested. As shown in **Table 4** and **Fig. 5**, good *Rs* values were obtained for most of the compounds.

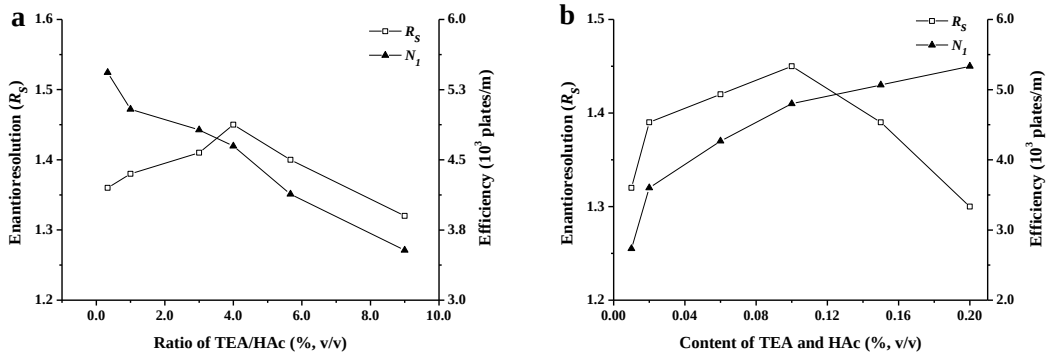
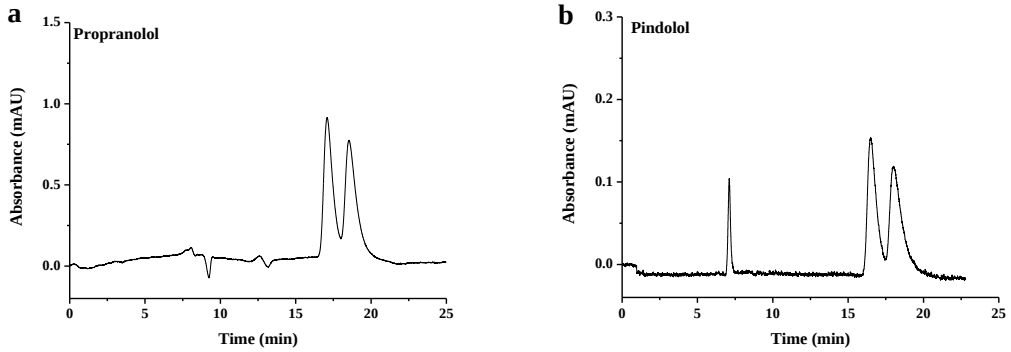


Fig. 4. Effect of the ratio (a) and content (b) of TEA/HAc on enantioresolution and column chromatographic efficiency for carteolol enantiomers in the polar organic-phase mode. Conditions: mobile phase: (a) MeOH/ACN/TEA/HAc (85/15/at the desired ratio of TEA and HAc, v/v/v/v); (b) MeOH/ACN/TEA/HAc (85/15/at the desired content of TEA and HAc, v/v/v/v); other experimental conditions are the same as in **Fig. 3**.



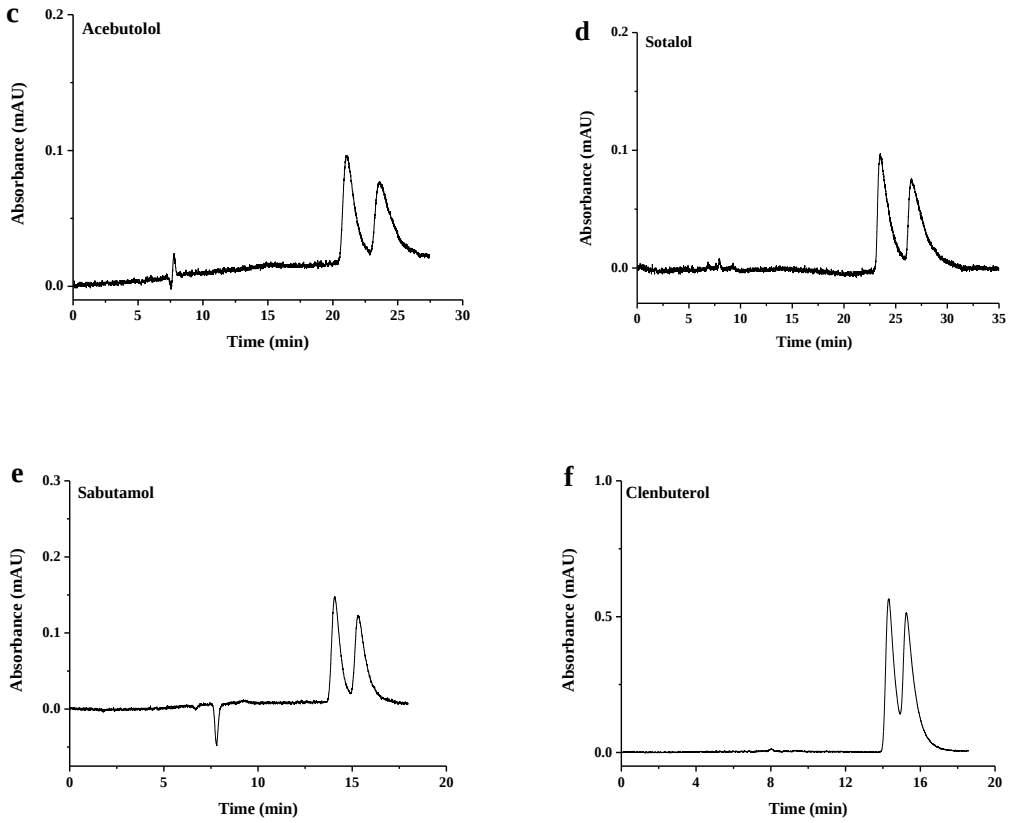


Fig. 5. Enantioseparation of racemic compounds in polar organic-phase mode. Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: MeOH/ACN/TEA/HAc (85/15/0.08/0.02, v/v/v/v); UV detection wavelength: 230 nm; total flow rate: 400 nL/min; injection volume: 20 nL.

Table 4. Enantioseparation of eight racemic compounds under the polar organic phase mode.

Sample	k_1	k_2	α	Rs	N_1 (m)	N_2 (m)
Carteolol	1.04	1.28	1.23	1.45	4500	4100
Propranolol	1.04	1.21	1.17	1.38	5100	4300
Acebutolol	1.26	1.48	1.17	1.43	4400	3900
Pindolol	1.07	1.23	1.15	1.32	3900	3200
Tertaolol	0.66	0.81	1.22	1.39	5500	4600
Sotalol	1.65	2.01	1.22	1.42	4100	3300
Clenbuterol	0.67	0.78	1.16	1.26	4600	4100
Salbutamol	0.74	0.88	1.18	1.47	4900	4100

Experimental conditions are the same as in **Fig. 4**.

3.3.2. Reversed phase mode

As reported in previous studies [14, 15], the basic compound carteolol was not enantio-resolved on the vancomycin functionalized monolith in the reversed phase elution mode where ACN was mainly used [23]. In our preliminary experiments, no enantioresolution of this analyte was observed employing similar conditions. Therefore, MeOH instead of ACN was chosen for the enantioseparation of carteolol to investigate the effect of MeOH concentration on the enantioseparation of carteolol. As shown in **Fig. 6a**, both retention factor (k_1) and enantioselectivity factor (α) increased by varying MeOH content in the mobile phase in the range 80-98 % (v/v) with the highest values at 98 % (v/v). This effect can be explained with a consequent stronger interaction of the studied enantiomers with vancomycin CSP because higher MeOH concentration combined with TEAA would lead to less nonselective hydrogen interaction [15]. As shown in **Fig. 6b**, the MeOH content also had a strong influence on the enantioresolution and column efficiency, and a slightly higher enantioresolution and column efficiency was obtained when the mobile phase contained 90% (v/v) MeOH. Hence, 90% (v/v) MeOH was selected as the optimum mobile phase for the enantioseparation of carteolol.

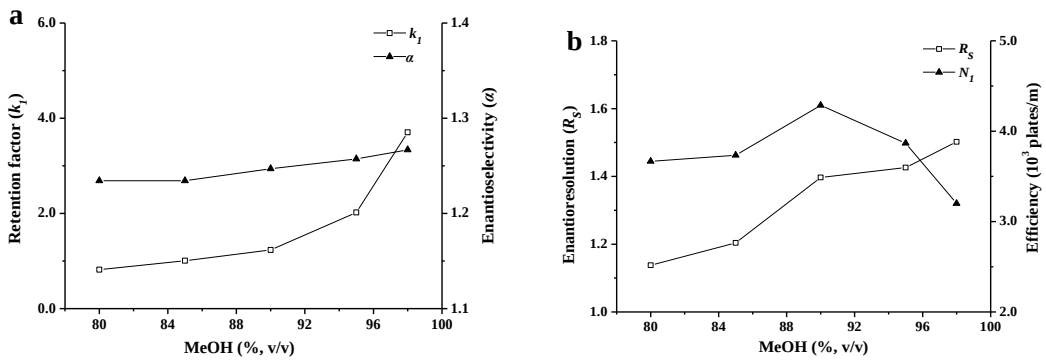


Fig. 6. Effect of the MeOH content on (a) retention factor (k_1) and enantioselectivity factor (α); (b) enantioresolution and column chromatographic efficiency for carteolol enantiomers in the reversed-phase mode. Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: 0.5% TEAA, pH=5.5/MeOH; UV detection wavelength: 230 nm; total flow rate: 400 nL/min; injection volume: 20 nL.

Due to the fact that in reversed phase mode the pH and content of buffer solution also played an important role for enantioseparation, they were investigated. **Fig. 7a** shows the effect of the buffer pH present in the mobile phase on enantioresolution and column chromatographic efficiency. Both parameters increased with increasing the buffer pH

value from 4.5 to 6.0 and the optimum pH value was 5.5. In order to improve the enantioseparation, various concentration of TEAA buffer were evaluated (**Fig. 7b**). A decrease of the enantioresolution factors with increasing TEAA buffer content can be observed, while the column chromatographic efficiency raised when the TEAA buffer content increased from 0.1% to 1% (v/v) and then decreased. As a compromise between enantioresolution and column efficiency, 0.5% TEAA (pH=5.5)/MeOH (10/90, v/v) was selected as the mobile phase. Carteolol enantiomers were baseline separated with *Rs* value of 1.59 as shown in **Fig. 8a**. Clenbuterol, salbutamol, acebutolol and several other β-blockers were also tested using 0.5% TEAA (pH=5.5)/MeOH (10/90, v/v) as the mobile phase. However, it was found that the enantioresolutions of these compounds were not satisfactory.

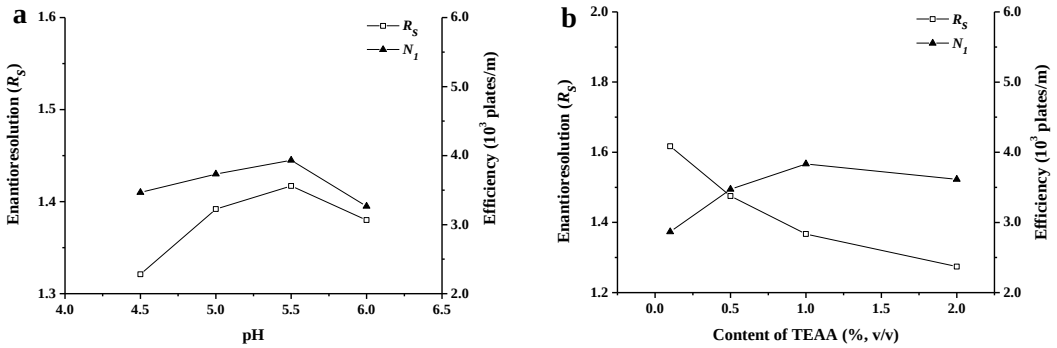


Fig. 7. Effect of TEAA buffer pH (a) and content (b) on enantioresolution and column chromatographic efficiency for carteolol enantiomers in the reversed-phase mode. Conditions: mobile phase: (a) 0.5% TEAA/MeOH (10/90, v/v); (b) TEAA, pH=5.5/MeOH (10/90, v/v); other experimental conditions are the same as in **Fig. 6.**

Colchicine was also tested under the above optimized conditions, unfortunately, no baseline enantioseparation was achieved. Therefore, a similar optimization process was performed. Under the optimized condition, i.e. 50 mM ammonium acetate (pH=5.5)/water/ACN (5/5/90, v/v/v), a baseline separation with R_s value of 2.92 was obtained for colchicine enantiomers (**Fig. 8b**). Due to the fact that thalidomide enantiomers were separated in previous reports using 0.2 % TEAA, pH 4.5/ACN (80:20, v/v) as mobile phase [23], these conditions were employed for the separation of thalidomide on the poly (ICNEML-vancomycin-*co*-EDMA) monolith column. It was found that the enantioresolution was still good (3.27) but the analysis time was too long (≥80 min). Thus, the mobile phase was re-optimized in which 0.5% TEAA buffer (pH=5.4)/ACN (70/30, v/v) offered the best output in terms of enantioresolution and analysis time (**Fig. 8c**). As shown in **Table 5**, carteolol, colchicine and thalidomide

enantiomers can be completely separated on the poly (ICNEML-vancomycin-*co*-EDMA) monolith column under the reversed-phase mode by nano-LC.

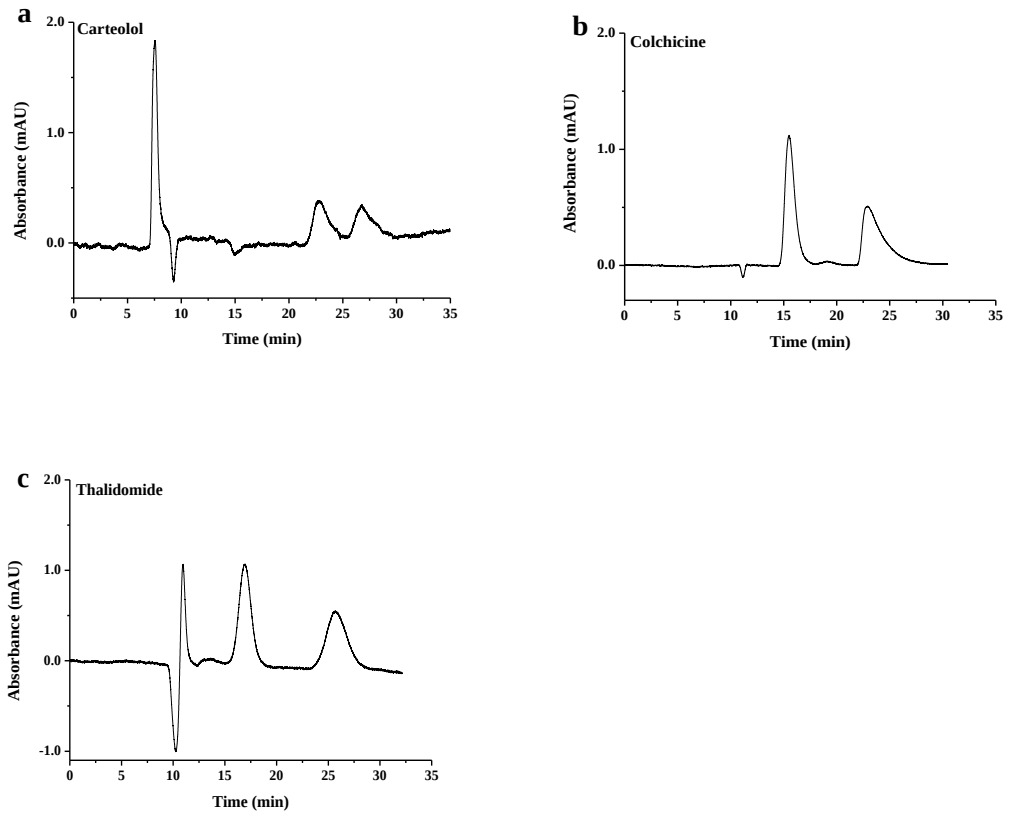


Fig. 8. Enantioseparation of (a) carteolol, (b) colchicine and (c) thalidomide in reversed-phase mode. Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: (a) 0.5% TEAA, pH=5.5/MeOH (10/90, v/v); (b) 50 mM ammonium acetate, pH=5.5/water/ACN (5/5/90, v/v/v); (c) 0.5% TEAA, pH=5.4/ACN (70/30, v/v); UV detection wavelength: 230 nm (a and c) or 243 nm (b); flow rate: 400 nL/min; injection volume: 20 nL.

Table 5. Enantioseparation of three racemic compounds under the reversed phase mode

Sample	k_1	k_2	α	*Rs*	N_1 (m)	N_2 (m)
Carteolol	2.18	2.72	1.24	1.59	3800	2500
Colchicine	0.41	1.08	2.62	2.92	6200	3300
Thalidomide	0.52	1.33	2.55	2.85	5100	4200

Experimental conditions are the same as in **Fig. 7**.

4. Conclusions

This study has demonstrated a novel and facile method to synthesize vancomycin functionalized organic polymeric monolith through a single-step approach, which

simplifies the fabrication of previous studies. The prepared monolith has been proven to possess large through-pores and a good mechanical stability. Satisfactory column permeability and good enantioselectivity were obtained on the optimum poly(ICNEML-vancomycin-*co*-EDMA) monolith. The mobile phase composition of different buffer pH, organic modifier content and buffer concentration which could influence the enantioseparation was further investigated both in the polar organic and reversed phase modes for enantioseparation of β-blockers. The vancomycin functionalized organic polymer monolith displayed baseline separation for most of the selected enantiomers in both chromatographic modes.

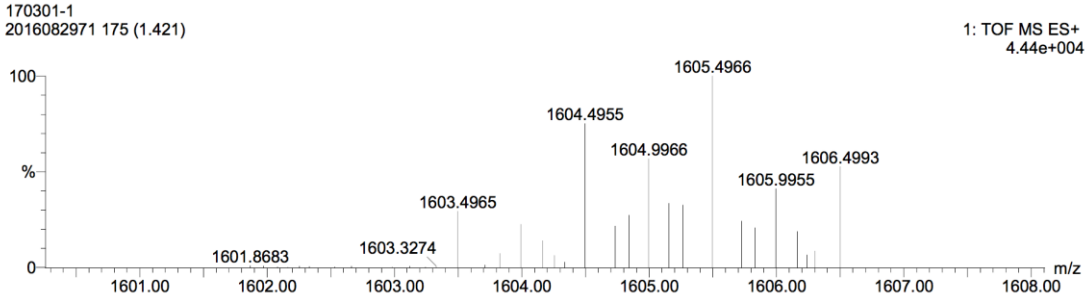
Appendix A.

Supplemental material for article 1.

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
349 formula(e) evaluated with 3 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-100 H: 0-100 N: 10-10 O: 0-50 Cl: 2-2
170301-1
2016082971 175 (1.421)



Minimum:				**-1.5**				
Maximum:		**5.0**	**10.0**	**50.0**				
Mass	**Calc. Mass**	**mDa**	**PPM**	**DBE**	**i-FIT**	**Norm**	**Conf(%)**	**Formula**
1603.4965	**1603.4963**	**0.2**	**0.1**	**35.5**	**278.3**	**0.388**	**67.87**	**C73 H85 N10 O27 Cl2**
	1603.4986	**-2.1**	**-1.3**	**4.5**	**280.2**	**2.230**	**10.75**	**C48 H97 N10 O45 Cl2**
	1603.4928	**3.7**	**2.3**	**13.5**	**279.5**	**1.543**	**21.38**	**C55 H93 N10 O40 Cl2**

Fig. S1. HR-ESI-MS of the molecular formula of ICNEML-vancomycin

Acknowledgements

M. L. Marina and E. Sánchez-López thank the Ministry of Economy and Competitiveness (Spain) for research project CTQ2013-48740-P. D. Xu and E. Sánchez-López thank the Comunidad of Madrid and European funding from FEDER program for their contracts (AVANSECAL-CM program, ref. S2013/ABI-3028). This work was also supported by the Science and Technology Planning Project of Guangdong Province, China (2016A040403056 and 2015A020211018) and the International Science & Technology Cooperation Program of Guangzhou, China (201807010022).

References

[1] S. Fanali, Nano-liquid chromatography applied to enantiomers separation, J. Chromatogr. A 1486 (2017) 20-34.

[2] B. Chankvetadze, Recent developments on polysaccharide-based chiral stationary phases for liquid-phase separation of enantiomers, J. Chromatogr. A 1269 (2012) 26-51.

[3] B. Chankvetadze, Monolithic chiral stationary phases for liquid-phase enantioseparation techniques, J. Sep. Sci. 33 (2010) 305-314.

[4] J.L. Guo, Y.J. Lin, Y. Xiao, J. Crommen, Z.J. Jiang, Recent developments in cyclodextrin functionalized monolithic columns for the enantioseparation of chiral drugs, J. Pharm. Biomed. Anal. 130 (2016) 110-125.

[5] N.W. Smith, Z.J. Jiang, Developments in the use and fabrication of organic monolithic phases for use with high-performance liquid chromatography and capillary electrochromatography, J Chromatogr A, 1184 (2008) 416-440.

[6] Q.X. Zhang, J.L. Guo, F. Wang, J. Crommen, Z.J. Jiang, Preparation of a β-cyclodextrin functionalized monolith via a novel and simple one-pot approach and application to enantioseparations, J. Chromatogr A, 1325 (2014) 147-154.

[7] Y.J. Lin, J.L. Guo, H. Lin, J.C. Wang, G. W. Somsen, J. Crommen, Z. Jiang, Effect of fabrication strategy on the enantioseparation performance of β-cyclodextrin-functionalized polymethacrylate monoliths: A comparative evaluation J. Sep. Sci. 40 (2017) 3754-3762.

[8] Q.Q. Wang, E. Sánchez-López, H. Han, H.H. Wu, P.J. Zhu, J. Crommen, M. L. Marina, Z.J. Jiang, Separation of N-derivatized di- and tri-peptide stereoisomers by micro-liquid chromatography using a quinidine-based monolithic column-Analysis of l-carnosine in dietary supplements, J. Chromatogr. A, 1428 (2016) 176-184.

[9] Q.Q. Wang, P.J. Zhu, M. Ruan, H.H. Wu, K. Peng, H. Han, G. W. Somsen, J. Crommen, Z.J. Jiang, Chiral separation of acidic compounds using an O-9-(tert-butylcarbamoyl) quinidine functionalized monolith in micro-liquid chromatography, J. Chromatogr. A 1444 (2016) 64-73.

[10] M. Lämmerhofer, E. C. Peters, C. Yu, F. Svec, J. M. J. Frechet, Chiral monolithic columns for enantioselective capillary electrochromatography prepared by copolymerization of a monomer with quinidine functionality. 1. Optimization of polymerization conditions, porous properties, and chemistry of the stationary phase, Anal. Chem. 72 (2000) 4614-4622.

[11] X.L. Dong, R.A. Wu, J. Dong, M.H. Wu, Y. Zhu, H.F. Zou, Polyacrylamide-based monolithic capillary column with coating of cellulose tris(3,5-dimethylphenyl-carbamate) for enantiomer separation in capillary electrochromatography, Electrophoresis, 29 (2008) 919-927.

[12] Y. Zheng, X. Wang, Y.B. Ji, Monoliths with proteins as chiral selectors for enantiomer separation, Talanta 91 (2012) 7-17.

[13] E. Machtejevas, A. Maruska, A new approach to human serum albumin chiral stationary phase synthesis and its use in capillary liquid chromatography and capillary electrochromatography, J. Sep. Sci. 25 (2002) 1-6.

[14] O. Kornysova, P.K. Owens, A. Maruska, Continuous beds with vancomycin as chiral stationary phase for capillary electrochromatography, Electrophoresis 22 (2001) 3335-3338.

[15] O. Kornysova, R. Jarmalaviciene, A. Maruska, A simplified synthesis of polymeric nonparticulate stationary phases with macrocyclic antibiotic as chiral selector for capillary electrochromatography, Electrophoresis 25 (2004) 2825-2829.

[16] T. Koide, K. Ueno, Enantiomeric separations of primary amino compounds by capillary electrochromatography with monolithic chiral stationary phases of chiral crown ether-bonded negatively charged polyacrylamide gels, J. Chromatogr. A 909 (2001) 305-315.

[17] B. Preinerstorfer, W. Lindner, M. Lämmerhofer, Polymethacrylate-type monoliths functionalized with chiral amino phosphonic acid-derived strong cation exchange moieties for enantioselective nonaqueous capillary electrochromatography and investigation of the chemical composition of the monolithic polymer, Electrophoresis 26 (2005) 2005-2018.

[18] D.W. Armstrong, Y. Tang, S. Chen, Y. Zhou, C. Bagwill, J. R. Chen, Macrocyclic antibiotics as a new class of chiral selectors for liquid chromatography, Anal. Chem. 66 (1994) 1473-1484.
[19] X.L. Dong, J. Dong, J.J. Ou, Y. Zhu, H.F. Zou, Preparation and evaluation of a vancomycin immobilized silica monolith as chiral stationary phase for CEC, Electrophoresis 28 (2007) 2606-2612.
[20] G.S. Ding, A.N. Tang, Capillary electrochromatographic separation of racemates on a norvancomycin-bonded monolithic column under reversed-phase conditions, J Chromatogr. A, 1208 (2008) 232-238.
[21] A.N. Tang, X.N. Wang, G.S. Ding, X.P. Yan, On-line preconcentration and enantioseparation of thalidomide racemates by CEC with the hyphenation of octyl and norvancomycin monoliths, Electrophoresis 30 (2009) 682-688.
[22] E. Pittler, M.G. Schmid, enantioseparation of dansyl amino acids by HPLC on a monolithic column dynamically coated with a vancomycin derivative, Biomed. Chromatogr. 24 (2010) 1213-1219.
[23] M.L. Hsieh, L.K. Chau, Y.S. Hon, Single-step approach for fabrication of vancomycin-bonded silica monolith as chiral stationary phase, J Chromatogr. A, 1358 (2014) 208-216.
[24] A. Berthod, U.B. Nair, C. Bagwill, D.W. Armstrong, Derivatized vancomycin stationary phases for LC chiral separations, Talanta 43 (1996) 1767.
[25] U.B. Nair, S.S.C. Chang, D.W. Armstrong, Y.Y. Rawjee, D.S. Eggleston, Elucidation of vancomycin's enantioselective binding site using its copper complex, J. V. McArdle, Chirality 8 (1996) 590.
[26] B. Xiong, L. Zhang, Y. Zhang, H. Zou, J. Wang, Capillary electrochromatography with monolithic poly (styrene-co-divinylbenzene-*co*-methacrylic acid) as the stationary phase, J. High Resol. Chromatogr. 23 (2000) 67-72.
[27] Z.J. Jiang, N.W. Smith, P.D. Ferguson, M.R. Taylor, Hydrophilic interaction chromatography using methacrylate-based monolithic capillary column for the separation of polar analytes, Anal. Chem. 79 (2007) 1243-1250.
[28] J. Lin, S. Liu, J. Lin, X. Lin, Z. Xie, Novel highly hydrophilic methacrylate-based monolithic column with mixed-mode of hydrophilic and strong cation-exchange interactions for pressurized capillary electrochromatography, J. Chromatogr. A 1218 (2011) 4671-4677.

[29] S.M. Zarcero, I. Sierra, Comparative HPLC methods for β-blockers separation using different types of chiral stationary phases in normal phase and polar organic phase elution modes. Analysis of propranolol enantiomers in natural waters, J. Pharm. Biomed. Anal. 62 (2012) 33-41.
[30] T.L. Xiao, R.V. Rozhkov, D.W. Armstrong, Separation of the enantiomers of substituted dihydrofurocoumarins by HPLC using macrocyclic glycopeptide chiral stationary phases, Anal. Bioanal. Chem. 377 (2003) 693-654.
[31] C. Karlsson, L. Karlsson, D.W. Armstrong, P.K. Owens, Evaluation of a vancomycin chiral stationary phase in capillary electrochromatography using polar organic and reversed-phase modes, Anal. Chem. 72 (2000) 4394-4401.

III. 2.

Preparation of teicoplanin functionalized organic silica hybrid monoliths as chiral stationary phase for nano-liquid chromatography

III.2. Preparation of teicoplanin functionalized organic silica hybrid monoliths as chiral stationary phase for nano-liquid chromatography

III.2.1. Preface

The development of silica-based and polymer-based monolithic columns has been limited due to the cumbersome and multi-step preparation method or their tendency to swell in organic solvents, respectively. In the case of particle loaded monolithic columns, the particles are prone to settling when they are used in high percentages, resulting in a poor dispersion of the chiral selector in the polymerization mixture. Recently, organic-silica hybrid monolithic columns have attracted great attention. On the one hand, the same simple preparation process as for polymer-based monolithic columns can be employed, and, on the other hand, they overcome the problem of swelling in organic solvents. Hence, these columns can be considered that combine the advantages of silica-based and polymer-based monoliths.

Teicoplanin is an important macrocyclic antibiotic. It has more active surface than other related glycopeptides due to the N-acyl hydrocarbon chain on the one glucosamine, and it has been widely used in enantioseparations. However, silica packed columns were mainly employed and only two teicoplanin aglycone (TAG) was prepared into a monolith by the particle-loaded approach. A poly(ICNEML-teicoplanin-*co*-EDMA) monolithic column was prepared by our research group; however, the column was unstable in polar organic phase mode. Therefore, the development of novel teicoplanin functionalized monolithic columns to overcome these problems is very meaningful. For this reason, a teicoplanin organic-silica hybrid monolithic column was developed in this PhD Thesis using a simple procedure which just needs mixing the hydrolysis solution (PEG, urea, HAc, TMOS and γ-MAPS), the copolymerization monomer (Tei-ICNEML) and the initiator (AIBN) by one-step, and then keeping them to react at 40 ºC for 12 h and at 60 ºC for 6 h.

III.2.2. Objectives

The specific objectives of this work were:

- To synthesize the teicoplanin functional monomer for the monolithic column.
- To prepare novel teicoplanin functionalized organic-silica hybrid monolithic columns by a single-step.

- To optimize the preparation approach to obtain good enantioresolution, enantioselectivity and column efficiency.
- To evaluate and characterize the chromatographic performance of the organic-silica hybrid monolithic columns.
- To optimize the mobile phase conditions and to apply the developed column to the enantioseparation of different enantiomers.

III.2.3. Results

The results obtained in this work are included in the following scientific article:

Article 2: *Single-step fabrication of a teicoplanin functionalized organic-silica hybrid monolith for enantioseparation by nano-liquid chromatography*

D. Xu, R. Luo, M. L. Marina, Z. Jiang

Submitted.

Article 2

Single-step fabrication of a teicoplanin functionalized organic-silica hybrid monolith for enantioseparation by nano-liquid chromatography

D. Xu, R. Luo, M. L. Marina, Z. Jiang

Submitted.

Abstract

A novel teicoplanin functionalized organic-silica hybrid chiral monolithic column was prepared by a facile single-step approach. The conditions for the preparation of the organic-silica hybrid chiral monolithic column were systematically optimized, including the amount of poly (ethylene glycol) (PEG) and urea, the tetramethyl orthosilicate (TMOS)/3-(trimethoxysilyl)-propylmethacrylate (γ-MAPS) ratio (v/v), the content of teicoplanin-2-isocyanatoethyl methacrylate (Tei-ICNEML) monomer, and the reaction temperature and time. The optimum column exhibited satisfactory efficiency, permeability and stability. In order to obtain satisfactory enantioseparation performance, reversed phase (RPM) and polar organic phase (POM) modes were employed, and different experimental conditions were optimized, such as buffer concentration and pH, amount of the organic solvent, concentration and ratio of TEA/HAc, and MeOH/ACN ratio. The stability of the Tei-ICNEML organic-silica hybrid monolithic column was also investigated in both POM and RPM separation modes. Then, this novel monolithic column was successfully applied to achieve the enantioseparation of amino alcohols, *N*-derivatized amino acids and mandelic acids. 15 out of 20 amino alcohols were baseline separated with good column efficiency in the POM mode, and 3 out of 5 mandelic acids and 5 out of 6 N-derivatized amino acids were baseline separated in the RPM mode

Keywords:

Teicoplanin organic-silica hybrid monolith, Enantioseparation, Nano-LC, Amino alcohols and amino acids, Mandelic acids.

1. Introduction

Enantiomeric separations have been a hot topic for a long time [1] and continue being a field of high interest nowadays [2]. In order to achieve short analysis times, high efficiency, and multi-separation modes, a series of chiral monoliths were developed based on cyclodextrins [3, 4], polysaccharides [5-7], macrocyclic antibiotics [8, 9], proteins [10, 11], ligand-exchange [12-14], molecular imprinting [15, 16], brush-type [17, 18] and ion-exchange [19-21]. They were widely used for the enantiomeric separation of a variety of compounds including amino acids [20, 22], small peptides [21, 22], non-steroidal anti-inflammatory drugs (NSAIDs) [23, 24], mandelic acids [3, 4], β-blockers [8, 25], among others. According to the different matrix materials, these chiral monoliths can be divided into four categories: (a) silica-based monolithic columns [26, 27], prepared by the sol-gel approach, including the hydrolysis, condensation, ageing, and baking. Although these monolithic columns exhibited a wide range of pH suitability, low backpressure in the high flow rate and significantly shorter run times, the cumbersome and multi-step preparation method makes the preparation cycle longer. In addition, in order to introduce the different monomers into the monolithic column surface, it is necessary a troublesome step to treat the bed. (b) polymer-based monolithic columns [3, 8, 22], prepared by mixing the monomers, porogens, cross-linker and initiator. This approach is not only simpler, but also the monomers that can be selected are more extensive than in the case of silica-based monolithic columns. However, these monolithic columns are prone to swelling in organic solvents and have low mechanical strength and poor stability due to the nature of the material. (c) Particle loaded monolithic columns, a new class of monolithic materials prepared by mixing particles with a polymer solution and simultaneously introducing them into the capillary. Particles can be modified by the chiral selector [12, 28, 29], or be the chiral selector itself [30, 31]. Compared with traditional monolithic columns, this approach based on fixing the particles to the monolithic material by physical methods opens up new ideas for the preparation of monolithic columns. However, the limitation of this approach is that the dispersion of particles in the polymerization mixture is poor because these materials are prone to settling when used in higher percentages. (d) Recently, more attention was turned to organic-silica hybrid monolithic columns [20, 32-34]. They were usually prepared by the sol-gel method using two different silanization reagents, functionalized trialkoxysilanes and tetraalkoxysilanes which are used as the cross-linker. These monolithic columns

overcome the problem of swelling in organic solvents and, due to the introduction of the functionalized trialkoxysilanes, they do not require a post-derivatization to modify the monomers in the monolith bed. Therefore, these columns can be considered that combine the advantages of the silica-based and polymer-based monoliths.

Teicoplanin is an important macrocyclic antibiotic. It has more active surface than other related glycopeptides due to the N-acyl hydrocarbon chain on the one glucosamine [35], and it has been used in the enantioseparation of dipeptides [36], NSAIDs [37], amino acids [38], and other compounds. By surveying the relevant keywords of "teicoplanin" and "chiral stationary phase" in SciFinder and Web of Science, there are more than one hundred articles related to this topic; however, they are almost based on silica packed columns, only a few works are based on monolithic columns [28, 29]. The first teicoplanin functionalized monolithic column was prepared by Schmid *et al.* [29]. The particle-loaded monolith was prepared by suspending the teicoplanin aglycone (TAG)-silica particles into the pre-polymerization solvent composed of methacrylamide, ammonium sulfate piperacyl diacrylamide, vinylsulfonic acid and phosphate buffer, then adding initiator (ammonium peroxodisulfate and *N,N,N'N'*-tetramethylethylenediamine) into the suspension, and immediately filling into the capillary. This column exhibited satisfactory enantioresolution for amino acids, glycyl-dipeptides and diastereomeric dipeptides. Later on, Gatschelhofer *et al.* [28] prepared a TAG-silica particle-loaded monolith via ring-opening metathesis polymerization. In this case, TAG-silica particles were suspended into the mixture of bicyclohept-2-ene, 1,4,4a,5,8,8a-hexahydro-1,4,5,8,exo,endo-dimethanonaphthalene, 2-propanol and toluene, then adding RuCl2(PCy3)2(CHPh) (Cy=cyclohexyl) as initiator and filling into the capillary whose surface had been modified by bicyclohept-2-en-5-ylmethyldichlorosilane. Finally, several glycyl-dipeptides were enantioseparated using different mobile phases. Since then, monolithic columns based on teicoplanin were not reported due to the bottleneck of the difficult allylation of the teicoplanin monomer. Recently, we successfully used 2-isocyanatoethyl methacrylate (ICNEML) to methacrylate vancomycin hydrochloride [8], and then Luo *et al.* [39] developed a poly (ICNEML-teicoplanin-*co*-EDMA) chiral monolithic column by mixing the methacrylated monomer, EDMA, methanol, DMSO and the initiator AIBN *via* single-step with *in-situ* polymerization in the capillary. However, the column was unstable in polar organic phase mode. Hence, the development of novel teicoplanin functionalized monolithic columns to overcome these problems is very meaningful.

In this work, a teicoplanin organic-silica hybrid monolithic column was prepared in a single-step by mixing the hydrolysis solution (poly (ethylene glycol) (PEG), urea, acetic acid (HAc), tetramethyl orthosilicate (TMOS) and 3-(trimethoxysilyl)-propylmethacrylate (γ-MAPS)), copolymerization monomer teicoplanin-2-isocyanatoethyl methacrylate (Tei-ICNEML) and initiator (2,2'-azobisisobutyronitrile (AIBN)). In order to obtain satisfactory enantioresolution, column efficiency and permeability, the preparation conditions were systematically optimized by adjusting the composition of the hydrolysis solution and the amount of monomer. The optimum monolithic column was applied to achieve the enantioseparation of chiral compounds under the polar organic-phase (POM) and reversed-phase (RPM) modes. Separation conditions were also optimized including buffer concentration and pH, and amount of organic solvent (RPM), and concentration and ratio of base/acid additive, and MeOH/ACN ratio (POM).

2. Materials and methods

2.1. Reagents and samples

Teicoplanin was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). Poly (ethylene glycol) (PEG) (MW=10000), tetramethyl orthosilicate (TMOS), urea, 2,2'-azobisisobutyronitrile (AIBN), 3-(trimethoxysilyl)-propylmethacrylate (γ-MAPS), ethylene dimethacrylate (EDMA), 2-isocyanatoethyl methacrylate (ICNEML), DMSO, methanol (MeOH), *N,N*-dimethylformamide (DMF), sodium hydroxide (NaOH), acetonitrile (ACN), triethylamine (TEA), acetic acid (HAc), pyridine and acetone were acquired from Aladdin Chemicals (Shanghai, China). Except antenolol and carteolol that were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), isoproterenol, metipranolol, acebutolol, sotalol, metoprolol, bisoprolol, alprenolol, propranolol, pindolol, betaxolol, oxprenolol, tertaolol, clenbuterol, salbutamol and talinolol were from Energy Chemical (Shanghai, China). The acidic standards including 4-hydroxyphenylglycolic acid, 3,4-difluorophenylacetic acid and 4-methoxyphenylglycolic acid were purchased from Alfa Aesar Chemicals Co., Ltd (Shanghai, China), while mandelic acid and 2-chloromandelic acid were purchased from Energy Chemical (Shanghai, China). Derivatized amino acids standards such as m-chlorobenzoyl chloride-alanine (*m*-ClB-alanine), *m*-chlorobenzoyl chloride-leucine (*m*-ClB-leucine), benzoyl chloride-leucine

(B-leucine), 3,5-dichlorobenzoyl chloride-alanine (3,5-DClB-alanine), and *p*-nitrobenzoyl-leucine (*p*-NB-leucine) chloride were synthesized in our laboratory [20], and N-acetyl-phenylalanine was bought from Energy Chemical (Shanghai, China).

Fused-silica capillaries (375 μm O.D. × 100 μm I.D.) were from Ruifeng Chromatography Ltd. (Hebei, China). Distilled water (DW) was purified using a Milli-Q system (Massachusetts, MA, USA). Polar organic mobile phases were set up by mixing the desired ratio of ACN and MeOH, and then adding various amounts of TEA and HAc. Reversed-phase mobile phases were prepared by mixing the corresponding ratio of MeOH and TEAA buffer which had been adjusted to the desired pH value by HAc. All mobile phases were subjected to filtration through a 0.22-μm membrane and sonication degas prior to be used.

2.2. Instrumentation

The nano-LC system was assembled in our laboratory, which consists of a DiNa nano gradient pump (Tokyo, Japan), a Shimadzu SPD-15C UV detector (Kyoto, Japan) with a lab-made on-column detection cell and a Valco four-port injection valve with 50 nL internal loop (Houston, TX, USA). The T-shape stainless steel connector (Houston, TX, USA) was adopted to link the pump to the valve through a flow split capillary (150 mm × 25 μm I.D) to decrease the flow rate and pressure inside the capillary. The thermally initiated co-polymerization was reacted in a Jinghong DK-S22 water bath (Shanghai, China). Scanning electron microscopy (SEM) experiments were performed with a Zeiss Gemini ultra-55 SEM (Deutschland, Germany) at an acceleration voltage of 5 kV. The pH values of buffer solutions were measured by a Sartorius PB-10 pH meter (Göttingen, Germany). All data acquisition and analysis were carried out with Unimicro TrisepTM Workstation 2003 (Shanghai, China), and all the figures were redrawn by Microcal Origin 8.5.

2.3. Preparation of the Tei-ICNEML organic-silica hybrid monolith columns

In order to anchor the bulk monoliths to the capillary inner wall, the fused-silica capillary was pretreated before use, including rinsed successively by NaOH (1.0 M) for 4 h, DW for 0.5 h, HCl (1.0 M) for 14 h, DW for 0.5 h, finally, dried at room temperature with nitrogen for 24 h [40]. The monomer Tei-ICNEML was synthesized according our previous works [8]. Then, the PEG (190 mg), urea (360 mg), 0.01 M HAc (2.5 mL), TMOS (0.9 mL) and γ-MAPS (0.25 mL) were mixed into a homogenous solution for

hydrolyzing at 0 ºC for 2.5 h. After the reaction, the hydrolyzed mixture solution (0.25 mL) was mixed with Tei-ICNEML monomer (15.0 mg) and AIBN (1.0 mg) via sonication of 10 min at room temperature, and then introduced into the desire long pretreated capillaries. The capillaries which both ends were sealed with rubber plugs were submerged into the water bath for incubating 12 h at 40 ºC and 6 h at 60 ºC, respectively. Finally, the unreacted PEG and other chemicals were removed by flushing the monolith column with H_2O and MeOH (**Fig. 1**).

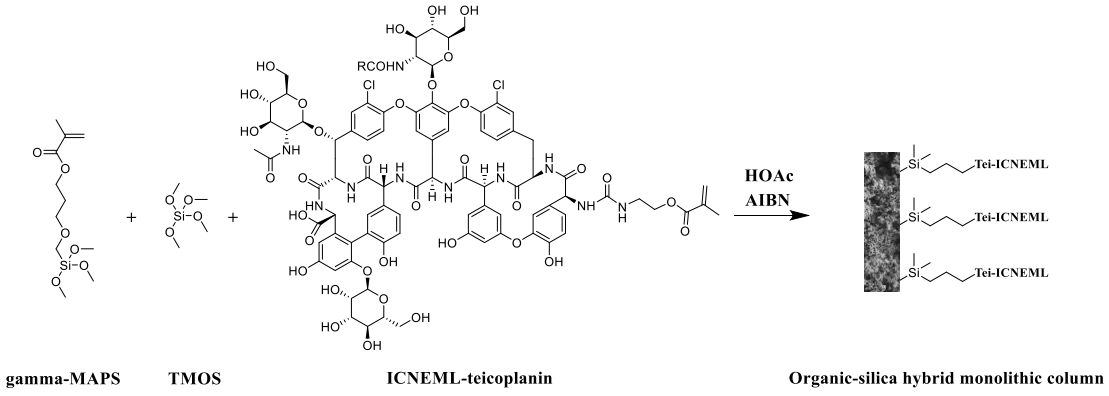


Fig. 1. Schematic representation of the fabrication of the Tei-ICNEML organic silica hybrid monolithic column.

3. Results and discussion

3.1. Preparation and characterization of the Tei-ICNEML organic-silica hybrid monolithic columns

In this work, Tei-ICNEML organic-silica hybrid monolithic columns were prepared via a single-step approach as reported previously by Lin *et al.* [40]. Because the Tei-ICNEML monomer exhibited good solubility in H_2O and DMSO, while it has poor solubility in MeOH, DMF and other higher alcohols, after a series of experiments, finally we chose the aqueous system to optimize the conditions for the preparation of the columns. In order to obtain the best monolithic column with satisfactory enantioseparation power, permeability and mechanical stability, the effect of several variables affecting the formation of the monolithic column was studied, including PEG and urea contents, TMOS/γ-MAPS ratio, amount of Tei-ICNEML monomer, and reaction temperature and time, as shown in **Table 1**.

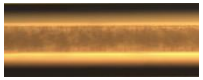
First, the effect of varying the amounts of PEG and urea was investigated when the TMOS/γ-MAPS ratio and the content of Tei-ICNEML monomer were kept at 18/5 and

15.0 mg, respectively. As can be seen in **Table 1**, the content of PEG was investigated from 210 mg to 180 mg (columns **C1** to **C3**). It can be observed that when increasing the PEG amount from 190 mg (column **C2**) to 210 mg (column **C1**), the morphology image of the monolithic column became transparent; conversely, when this content decreased from 190 mg (column **C2**) to 180 mg (column **C3**), the morphology image of the monolithic column changed to slack. This phenomenon also occurred when the amount of urea was increased or decreased, as can be seen in column **C5** (350 mg), column **C2** (360 mg) and column **C4** (370 mg). Column **C2** exhibited the most homogeneous morphology and the best permeability when the content of urea was 360 mg. Therefore, 190 mg PEG and 360 mg urea were selected for the following studies.

Second, the effect of the TMOS/γ-MAPS ratio (v/v) was investigated when the contents of PEG, urea and teicoplanin-ICNEML monomer were kept constant. As shown in **Table 1**, when decreasing the TMOS/γ-MAPS ratio from 9:2 to 3:1 (column **C6** (9:2), column **C2** (18:5) and column **C7** (3:1)), the morphology images of the hybrid monolith changed from slack to transparent. When the TMOS/ γ-MAPS ratio (v/v) was 18:5, the hybrid monolith exhibited the most homogeneous morphology and the best permeability. Hence, 0.9 mL TMOS and 0.25 mL γ-MAPS were selected for the next experiments.

Third, the effect of the content of the Tei-ICNEML monomer was investigated. This is another important parameter, because it not only affected the morphology, but also is the chiral selector. Therefore, different contents of the Tei-ICNEML monomer were assayed from 10 mg to 20 mg. It was found that when the content of the Tei-ICNEML monomer decreased from column **C2** (15 mg) to column **C8** (10 mg), the morphology images of the hybrid monolith became semitransparent, while when increasing the content from **C2** (15 mg) to column **C9** (20 mg), the morphology images of the hybrid monolith became slack. In addition, high contents of the Tei-ICNEML monomer caused that the pre-condensation mixture changed to gel quickly which made difficult to introduce it into the capillary. Finally, 15 mg Tei-ICNEML monomer were selected for next experiments.

Table 1. Optimization of the conditions for the preparation of the Tei-ICNEML organic silica hybrid monolithic columns.

Column	PEG (mg)	Urea (mg)	TMOS/ γ-MAPS (μL, v/v)	Teicoplanin (mg)	Temperature and Time (°C, h)	Backpressure (MPa)	Morphology
C1	210	360	900/250	15.0	40 °C, 12 h; 60 °C, 6 h	Too hard	
C2	190	360	900/250	15.0	40 °C, 12 h; 60 °C, 6 h	2.1	
C3	180	360	900/250	15.0	40 °C, 12 h; 60 °C, 6 h	0.1	
C4	190	370	900/250	15.0	40 °C, 12 h; 60 °C, 6 h	Too hard	
C5	190	350	900/250	15.0	40 °C, 12 h; 60 °C, 6 h	0.3	
C6	190	360	900/200	15.0	40 °C, 12 h; 60 °C, 6 h	Too hard	
C7	190	360	900/300	15.0	40 °C, 12 h; 60 °C, 6 h	Detached	
C8	190	360	900/250	10.0	40 °C, 12 h; 60 °C, 6 h	Too hard	
C9	190	360	900/250	20.0	40 °C, 12 h; 60 °C, 6 h	Detached	
C10	190	360	900/250	15.0	35 °C, 12 h; 60 °C, 6 h	Detached	
C11	190	360	900/250	15.0	45 °C, 12 h; 60 °C, 6 h	Too hard	

Conditions: column dimensions: 10 cm × 100 μm I.D.; mobile phase: MeOH/ACN/TEA/HAc (80/20/0.03/0.055, v/v/v/v); UV detection wavelength: 225 nm; total flow rate: 20 μL/min; injection volume: 20 nL; sample: Metoprolol.

The effect of the reaction temperature and time was also investigated since not only the skeleton formation was affected by these parameters but they also controlled the generation of pore and polymerization. According to that previously reported [40], a temperature of 40 ºC and a reaction time of 12 h were firstly selected for the preparation of the column. However, under these conditions, there was no way to get a monolithic column with enantioseparation ability. Nevertheless, the problem was well solved when the temperature was continuing to raised to 60 ºC for 6 h. It can be interpreted that the skeleton was formed at 40 ºC, while the polymerization occurred at 60 ºC. It was found that the hybrid monolith was slack when 35 ºC (column **C10**) was selected, and the monolith could be detached from the capillary inner wall, however, the permeability became poor when increasing the temperature to 45 ºC (column **C11**). Hence, 40 ºC during 12 h followed by 60 ºC during 6 h were selected for the preparation.

According to these optimization experiments, the hybrid monolith mixture was mixed with Tei-ICNEML monomer (15 mg), AIBN (1.0 mg) and 0.25 mL hydrolysis solution including PEG (190 mg), urea (360 mg), 0.01 M HAc (2.5 mL), TMOS (0.9 mL) and γ-MAPS (0.25 mL), after incubating at 40 ºC for 12 h and 60 ºC for 6 h. The morphology of the optimized Tei-ICNEML organic-silica hybrid monolithic column (column **C2**) was evaluated by scanning electron microscopy (SEM). As shown in **Fig. 2**, the SEM images indicated that the column **C2** has a morphology of a continuous skeleton and large through-pores, and the monolithic rod was tightly anchored on the inner wall of the capillary column.

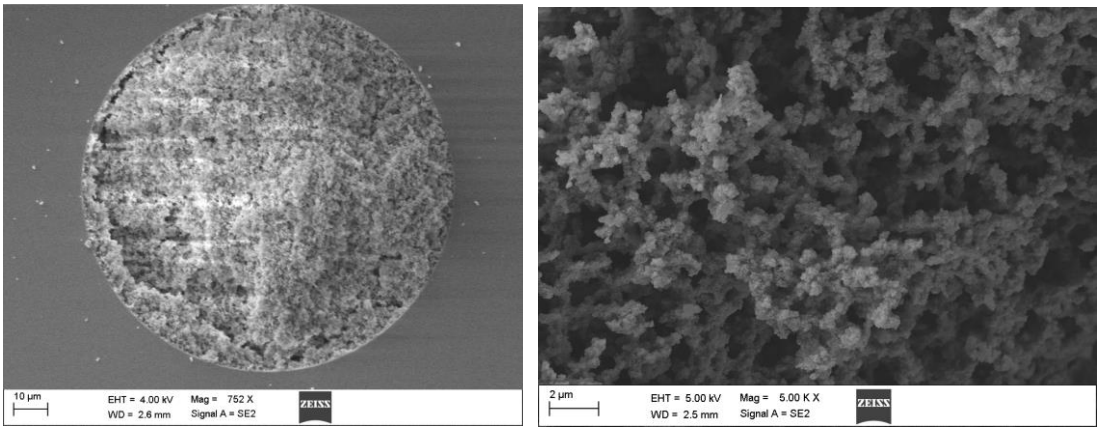


Fig. 2. Different magnifications of SEM images of the Tei-ICNEML organic silica hybrid monolithic column.

3.2. Permeability, mechanical stability and reproducibility of the Tei-ICNEML organic-silica hybrid monolithic columns

Three different chromatographic conditions were selected to investigate the permeability and mechanical stability of the column, including 100% MeOH, 100% ACN and 50/50 (v/v) H_2O/ACN. The permeability *K* value of the hybrid monolithic column **C2** can be calculated according to the following equation [8, 22]:

$$K = \frac{u\eta L}{\Delta P}$$

where *u* represents the mobile phase linear velocity, L the column length, ΔP the pressure drop across the column, and η the dynamic viscosity of the eluent. As can be seen in **Table S1**, the *K* values for the hybrid monolithic column **C2** were 1.76×10^{-14}, 1.81×10^{-14} and 0.82×10^{-14} m^2 when MeOH, ACN and 50/50 (v/v) H_2O/ACN were used as the mobile phase, respectively. It was found that the Tei-ICNEML organic-silica hybrid monolithic column didn't swell and shrink in the different polarities solvents due to their quite similar permeability values.

The mechanical stability was investigated by studying the linearity between the linear velocity and the backpressure under the different conditions. As can be seen in **Fig. S1**, when increasing the linear velocity, the total backpressure increased, and the linear correlation coefficent *r* values for the linear velocity and the backpressure in 100% MeOH, 100% ACN and 50/50 (v/v) H_2O/ACN were 0.9996, 0.9976, 0.9982, respectively, which indicated that the Tei-ICNEML organic-silica hybrid monolithic column had good mechanical stability under the different polarities solvents.

The reproducibility of the Tei-ICNEML organic-silica hybrid monolithic column was thoroughly evaluated by calculating the RSD values for k_1, k_2, α and *Rs* for metoprolol under the polar organic phase mode (MeOH/ACN/TEA/HAc (85/15/0.05/0.05, v/v/v/v)) (**Table S2**). The RSD values for column-to-column (n=3) for k_1 and k_2 were 3.26% and 3.97, respectively. The run-to-run repeatability (n=6) for k_1 and k_2 was also adequate with RSD values of 0.59% and 0.63%, in addition to day-to-day reproducibility (n=3) with RSD values of 1.30% and 1.52%, respectively. RSD values for α and *Rs* were also satisfactory (≤ 4.15%). From these data, it can be derived that the Tei-ICNEML organic-silica hybrid monolithic column has a satisfactory reproducibility.

3.3. Application for enantioseparation of racemate standards

3.3.1. Polar organic phase mode

According to our previous reports and other literature [8, 20], a polar organic phase mode was selected consisting of an organic solvent (MeOH and ACN), and acid-base additives (TEA and HAc). As reported previously, the hydrogen bonding, charge-charge and π-π interaction would be affected the enantioseparation due to the different composition of the mobile phase [8, 20]. In order to further investigate the enantioseparation on the Tei-ICNEML organic-silica hybrid monolithic column in this polar organic phase mode, propranolol was used as the test analyte.

The influence of TEA/HAc ratio on the enantioresolution and efficiency was firstly investigated by varying this ratio from 1:9 to 9:1, keeping constant the total concentration of TEA and HAc, and the MeOH/ACN ratio. As can be seen in **Fig. 3a** and **S2a**, when increasing the TEA/HAc ratio from 1:9 to 1:1, the enantioresolution of the test analyte increased and reached the maximum values, while the enantioresolution decreased when increasing the TEA/HAc ratio from 1:1 to 9:1. It can be interpreted that when decreasing the concentration of CH_3COO^- when increasing the TEA/HAc ratio from 1:9 to 9:1, the less CH_3COO^- concentration leaded to stronger interaction between the CSP and the test analyte, due to hydrogen bonding that played a dominant role in this polar organic-phase mode. However, when increasing the concentration of TEA (TEA/HAc ratio from 1:1 to 9:1), the interaction between the CSP and the test analyte was weakened, because analyte ionization was also weakened under these conditions. Moreover, as shown in **Fig. 3a**, the column efficiency increased when the TEA/HAc ratio increased from 1:9 to 9:1. From these results, a TEA/HAc ratio of 1:1 was selected for the following experiments, as a compromise between enantioresolution and column efficiency.

Then, the effect of the total concentration of the acid-basic additives (TEA and HAc) on the enantioresolution, efficiency and retention factor was investigated. As can be seen in **Fig. 3b**, the enantioresolution of propranolol had the maximum values when the total concentration of TEA and HAc was 0.02%. However, the retention factors increased when the total concentration decreased from 0.14% to 0.02% (**Fig. S2b**), because the additives weakened the interaction between the CSP and analytes. In addition, the highest column efficiency occurred at a 0.06% (**Fig. 3b**). Considering the results obtained for the enantioresolution, retention time and column efficiency, a 0.06%

total concentration of TEA and HAc was chosen for further experiments.

Last, the effect of the MeOH/ACN ratio was evaluated when the TEA/HAc ratio and the total concentration of TEA and HAc were kept constant at 1:1 and 0.06%, respectively. As shown in **Fig. S2c**, an increase in the MeOH/ACN ratio from 60:40 to 90:10 (v/v) originated a decrease in the retention factor. This is because the nonselective hydrogen bonding is weakened between the CSP and the analyte due to the increased content of MeOH in the mobile phase. As shown in **Fig. 3c**, the highest column efficiency and satisfactory enantioresolution was observed at a 80:20 (v/v) MeOH/ACN ratio.

As shown in **Table 2**, 20 amino alcohol enantiomers were separated under the optimal mobile phase conditions: MeOH/ACN/TEA/HAc (80:20:0.03:0.03, v/v/v/v), 15 out of them were baseline separated with good column efficiency. **Fig. 4** shows some of the enantiomeric separations achieved for these compounds under the optimized conditions.

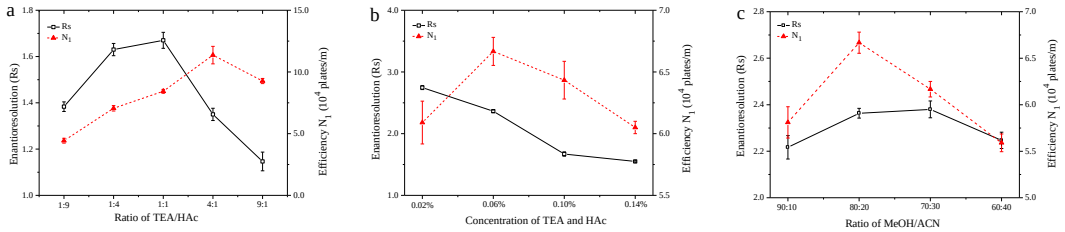


Fig. 3. Effect of (a) TEA/HAc ratio (keeping the total concentration of TEA and HAc at 0.1%), (b) total concentration of TEA and HAc (keeping the TEA/HAc ratio at 1:1), and (c) MeOH/ACN ratio, on column chromatographic efficiency and enantioresolution for propranolol in the polar organic phase mode. Conditions: column dimensions: 10 cm × 100 μm I.D.; mobile phase: (a) MeOH/ACN/TEA/HAc (80/20/desired ratio of TEA and HAc, v/v/v/v); (b) MeOH/ACN/TEA/HAc (80/20/desired total concentration of TEA and HAc, v/v/v/v); (c) MeOH/ACN/TEA/HAc (desired MeOH and ACN ratio/0.03%/0.03%, v/v/v/v); UV detection wavelength: 225 nm; total flow rate: 20 μL/min; injection volume: 50 nL; sample: propranolol.

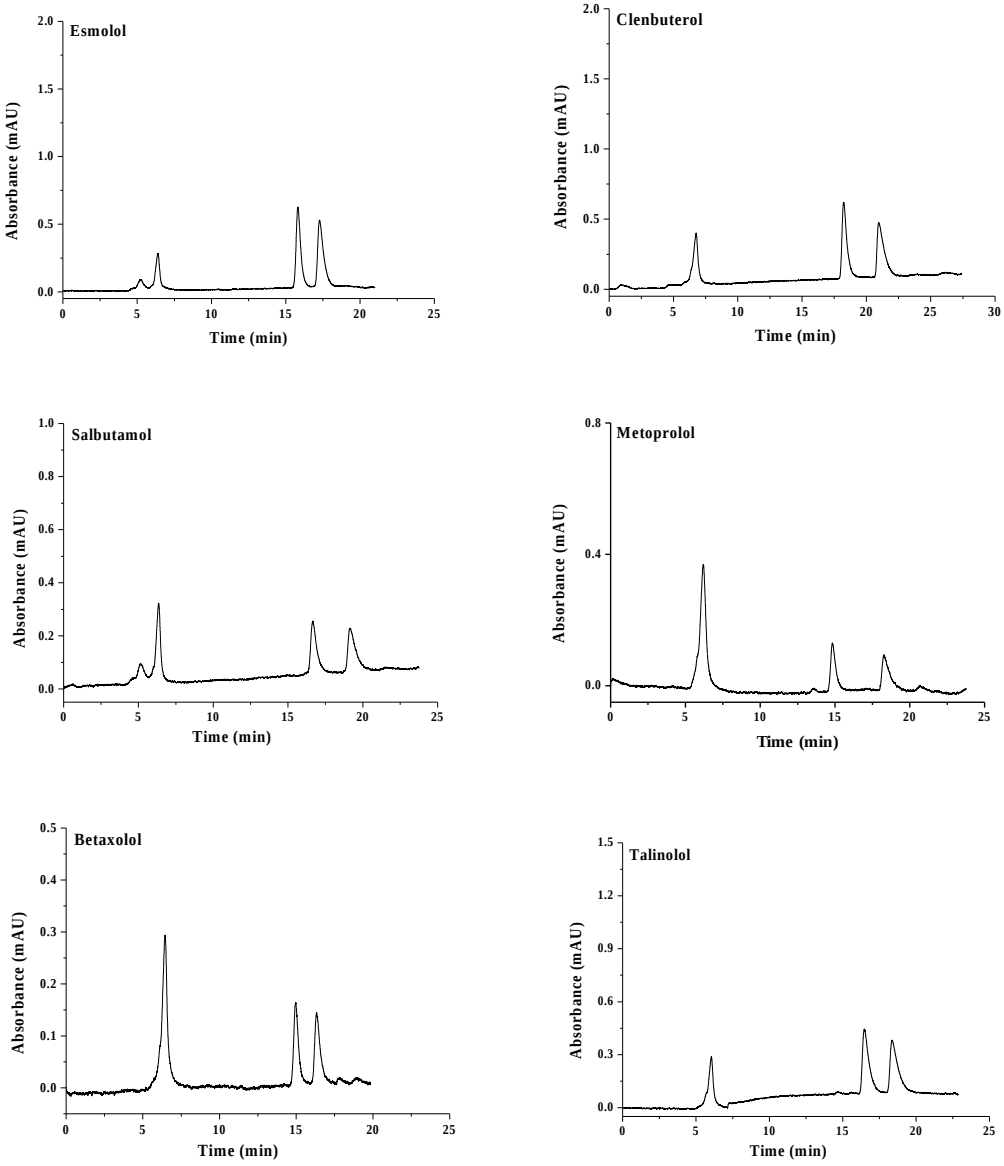


Fig. 4. Enantioseparation of racemic standards under the polar organic phase mode. Conditions: column dimensions: 10 cm × 100 μm I.D.; mobile phase: MeOH/ACN/TEA/HAc (80/20/0.03/0.03, v/v/v/v); other experimental conditions as in **Fig. 3**.

Table 2. Enantioseparation of racemic standards in polar organic phase mode.

Sample	k_1	k_2	α	Rs	N_1 (m)	N_2 (m)
Alprenolol	1.47	1.71	1.17	2.23	77000	65000
Atenolol	2.67	3.06	1.15	1.61	38000	26000
Esmolol	1.47	1.70	1.15	2.22	98000	72000
Betaxolol	1.32	1.53	1.16	2.07	84000	71000
Acebutolol	1.77	1.95	1.10	1.36	72000	54000
Clenbuterol	1.70	2.10	1.24	3.17	83000	56000
Metipranolol	1.30	1.50	1.16	1.67	56000	45000
Salbutamol	1.62	2.02	1.24	2.74	57000	43000
Sotalol	2.05	2.37	1.16	1.98	53000	47000
Talinolol	1.73	2.04	1.18	2.06	51000	42000
Tertatolol	1.72	2.01	1.17	2.29	80000	62000
Oxprenolol	1.39	1.53	1.10	1.45	87000	79000
Pindolol	1.75	2.03	1.16	1.75	63000	51000
Carazolol	1.91	2.16	1.13	1.48	67000	54000
Bisoprolol	1.19	1.38	1.16	1.88	88000	79000
Carvedilol	1.61	1.72	1.07	0.51	25000	19000
Isoproterenol	1.63	2.05	1.26	1.38	21000	15000
Metoprolol	1.39	1.95	1.40	4.57	72000	50000
Propranolol	1.76	2.07	1.18	2.38	67600	66000
Carteolol	2.26	2.71	1.20	2.24	41000	32800

Experimental conditions are the same as in **Fig. 4**.

3.3.2. Reversed phase mode

Due to the good enantioresolutions reported for acidic enantiomers on the teicoplanin

functionalized polymer-based monolithic and silica packed column under the reversed phase mode [28, 38], we selected mandelic acid as the test analyte to investigate the potential of the Tei-ICNEML organic-silica hybrid monolithic column in reversed phase mode. In our preliminary experiments, two different organic modifiers, MeOH and ACN, were investigated. As MeOH exhibited better enantioresolution than ACN, MeOH was selected as the organic modifier for the following experiments. In addition, the sample solvent had a great influence on the peak shape. It is not difficult to understand from previous reports [41, 42]. Usually, organic solvents are used to improve the solubility of sample components in the reversed phase mode, if the sample solvent is stronger than the mobile phase, it will cause distortions of the peak shape. In order to overcome this problem, all the samples were firstly dissolved in MeOH then diluted with the mobile phase.

Then, the effect of the pH of the TEAA buffer was investigated when the concentration of TEAA and MeOH were kept constant at 1% and 30%, respectively. As shown in **Fig. S3a**, when the pH of the TEAA buffer increased from 4.0 to 6.0, α increased, while k_1 decreased. This behavior can be ascribed to that the electrostatic repulsion interactions enhanced with the pH values changing. However, the enantioresolution and column efficiency reached their maximum value at pH=5.5. Therefore, a pH=5.5 was chosen for the following experiments (**Fig. 5a**).

Another important parameter is the concentration of the TEAA buffer in the mobile phase, which was investigated when the TEAA buffer pH and the content of MeOH were kept constant at 5.5 and 30%, respectively. As can be seen in **Fig. S3b**, k_1 increased when the concrentration of the TEAA buffer increased from 0.1 to 2%, because the electrostatic repulsion interaction between the stationary phase and analyte was competitively inhibited as the concentration of the TEAA buffer gradually increased. As can be seen in **Fig. 5b**, the enantioresolution had the highest value when the buffer concentration was 1%. Consequently, 1% of the TEAA buffer was selected for the further experiments.

Fig. 5c and **S3c** show the effect of the MeOH content on the enantioseparation. Based on the results of the previous optimization, the pH and concentration of the TEAA buffer were kept constant at 5.5 and 1%. When increasing the MeOH content from 15% to 40%, the elution ability of the mobile phase increased, and the retention factor of the analyte gradually became weak. Conversely, the enantioselectivity decreased when the MeOH content increased. However, when the content of MeOH was 30%, the column

efficiency and enantioresolution reached a maximum value at the same time. Hence, 30% MeOH was chosen for the next experiments.

Under the optimized conditions, 1% TEAA, pH=5.5/MeOH (70:30, v/v), eleven acidic chiral compounds including five mandelic acid derivatives and six N-derivatized amino acids were analyzed. As can be seen in **Fig. 6** and **Table 3**, all of the N-derivatized amino acids and three mandelic acid compounds were baseline separated.

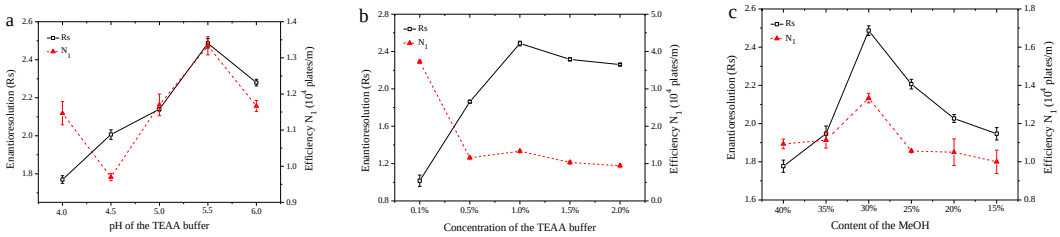


Fig. 5. Effect of (a) pH and (b) concentration of TEAA buffer, and (c) content of MeOH, on column chromatographic efficiency and enantioresolution for mandelic acid in the reversed phase mode. Conditions: column dimensions: 10 cm × 100 μm I.D.; mobile phase: (a) 1% TEAA, (at desired pH values)/MeOH (70/30, v/v); (b)TEAA (at desired concentration), pH=5.5/MeOH (70/30, v/v); (c) 1% TEAA, pH=5.5/MeOH (at desired content); UV detection wavelength: 220 nm; total flow rate: 10 μL/min; injection volume: 50 nL; sample: mandelic acid.

4. Conclusion

A novel Tei-ICNEML functionalized organic-silica hybrid monolithic column was successfully developed through a “single-step” strategy. By optimizing the parameters affecting the formation of the monolithic column, the optimum chiral organic-silica hybrid monolithic column exhibited good mechanical stability, permeability, and satisfactory enantioselectivity. In order to obtain the optimum chromatographic separation, two different mobile phase systems such as polar organic phase and reversed phase were investigated, then, all of the N- derivatized amino acids and three mandelic acids were baseline separated in the reversed phase mode, and 15 out of 20 amino alcohols were baseline separated with good column efficiency in the polar organic phase mode. Compared with the polymer based teicoplanin functionalized monolithic column (poly(ICNEML-Teicoplanin-*co*-EDMA)) [39], the novel hybrid monolithic column exhibited higher column efficiency and enantioresolution under the POM and RPM except for mandelic acids. Moreover, it had almost the same selectivity for the amino alcohols as poly(ICNEML-Teicoplanin-*co*-EDMA) in POM, while it exhibited higher selectivity for the N- derivatized amino acids in the RPM (as shown in **Fig. S4**).

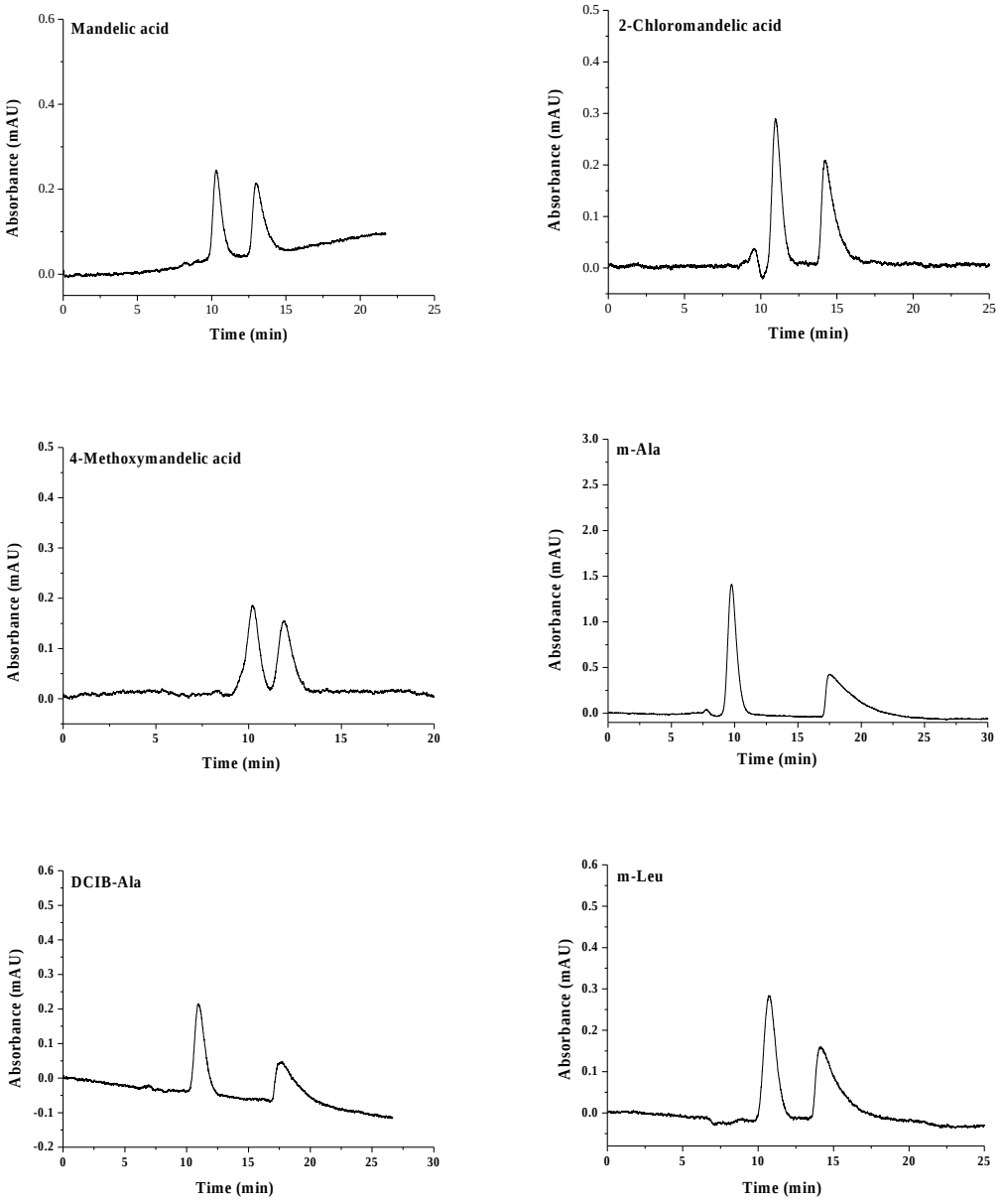


Fig. 6. Enantioseparation of racemic standards under the polar organic phase mode. Conditions: column dimensions: 10 cm × 100 μm I.D.; mobile phase: 1% TEAA， pH=5.5/MeOH(70/30, v/v); other experimental conditions as in **Fig. 5**.

Table 3. Enantioseparation of racemic standards in reversed phase mode.

Sample	k_1	k_2	α	Rs	N_1 (m)	N_2 (m)
4-Hydroxyphenylglycolic acid	0.07	0.25	3.63	1.53	9100	8500
4-Methoymandelic acid	0.08	0.26	3.24	1.42	10300	8700
3,4-Difluoromandelic acid	0.14	0.30	2.06	1.36	9700	7500
2-Chloromandelic acid	0.14	0.48	3.32	2.51	13300	11500
mandelic acid	0.18	0.56	3.10	2.29	12200	10100
N-Acetyl-phenylalanine [a]	0.14	0.48	3.42	2.43	8400	5700
3,5-DClB-Alanine [b]	0.19	0.92	4.80	2.79	6200	3800
m-ClB-Alanine [b]	0.25	1.25	4.91	3.12	6800	3100
m-ClB-Leucine [b]	0.18	0.57	3.08	2.36	6500	4300
p-NB-Leucine [b]	0.08	0.38	4.60	1.76	6900	5900
B-Leucine [b]	0.19	0.42	2.19	1.43	7600	5200

Experimental conditions are the same as in **Fig. 6**.

Appendix B.

Supplementary material for article 2.

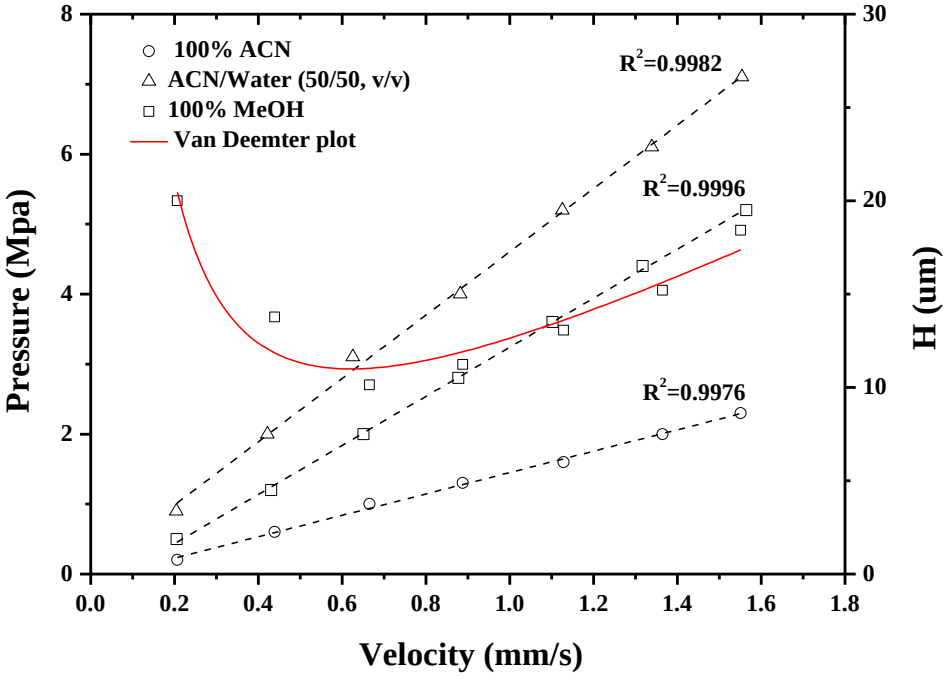


Fig. S1. Dependence of backpressure on linear velocity and Van Deemter plots for the Tei-ICNEML organic silica hybrid monolith. Experimental conditions: column dimensions: 10 cm × 100 μm I.D.; mobile phase for Van Deemter plots test: 100% ACN, UV detection wavelength: 214 nm; injection volume: 50 nL; samples: Toluene (100% ACN or 100% MeOH as mobile phase) and thiourea (H_2O/ACN (50/50, v/v) as mobile phase).

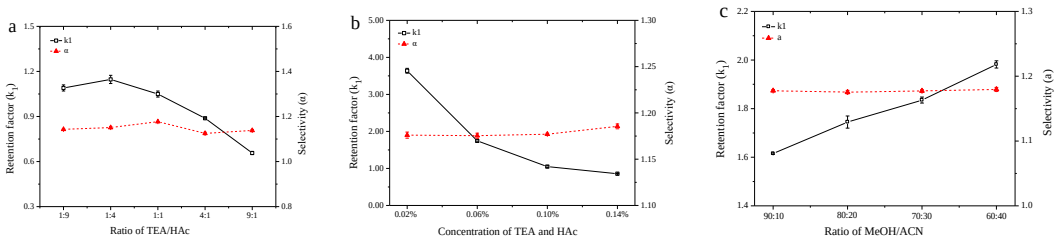


Fig. S2. Effect of (a) TEA/HAc ratio (keeping the total concentration of TEA and HAc at 0.1%), (b) concentration of TEA and HAc (keeping the TEA/HAc ratio at 1:1), and (c) MeOH/ACN ratio on the retention factor and selectivity for propranolol in the polar organic phase mode. Conditions: column dimensions: 10 cm × 100 μm I.D.; mobile phase: (a) MeOH/ACN/TEA/HAc (80/20/desired TEA and HAc ratio, v/v/v/v); (b) MeOH/ACN/TEA/HAc (80/20/ desired concentration of TEA and HAc, v/v/v/v); (c) MeOH/ACN/TEA/HAc (desired ratio of MeOH and ACN/0.03%/0.03%, v/v/v/v); UV detection wavelength: 225 nm; total flow rate: 20 μL/min; injection volume: 50 nL.

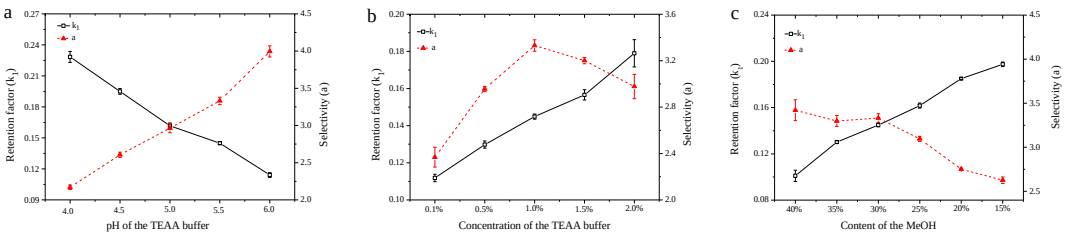


Fig. S3. Effect of (a) pH and (b) concentration of TEAA buffer, and (c) content of MeOH on the retention factor and selectivity for mendelic in the reversed phase mode. Conditions: column dimensions: 10 cm × 100 μm I.D.; mobile phase: (a) 1% TEAA, (at desired pH values)/MeOH (70/30, v/v); (b)TEAA (at desired concentration), pH=5.5/MeOH (70/30, v/v); (c) 1% TEAA, pH=5.5/MeOH (at desired content); UV detection wavelength: 220 nm; total flow rate: 10 μL/min; injection volume: 50 nL.

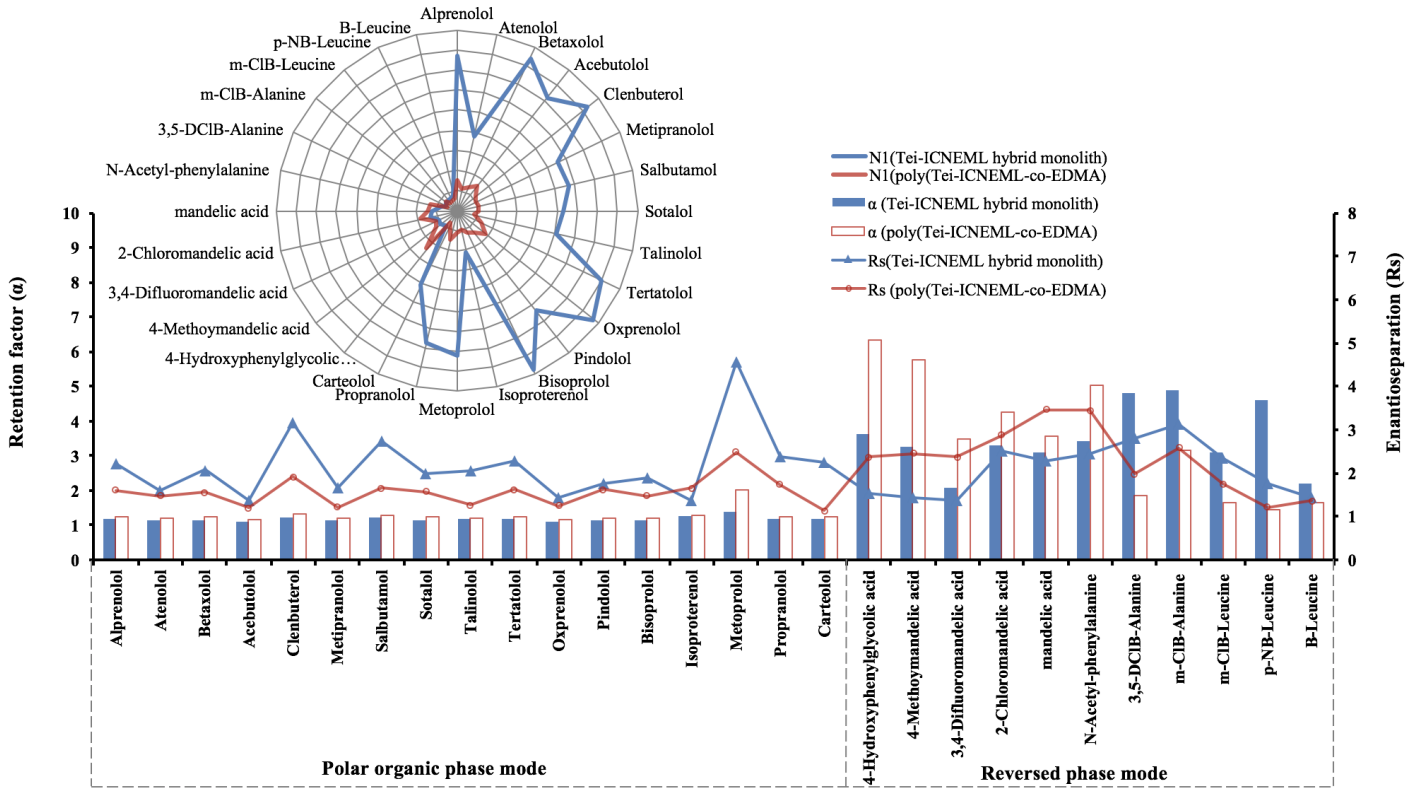


Fig. S4. Comparison of column chromatographic efficiency, enantioresolution and selectivity with the poly (Tei-ICNEML-*co*-EDMA) under the polar organic phase and reversed phase mode. Experimental conditions for Tei-ICNEML organic-silica hybrid monolith: POM: MeOH/ACN/TEA/HAc (80/20/0.03/0.03, v/v/v/v); UV detection wavelength: 225 nm; total flow rate: 20 μL/min; injection volume: 50 nL; RPM: 1% TEAA, pH=5.5/MeOH(70/30, v/v); UV detection wavelength: 220 nm; total flow rate: 10 μL/min; injection volume: 50 nL. The conditions for poly (Tei-ICNEML-*co*-EDMA) monolith: POM: MeOH/ACN/TEA/HAc (80/20/0.03/0.055, v/v/v/v); flow rate: 1200 nL/min; UV detection wavelength: 225 nm; injection volume: 20 nL; RPM: 1% TEAA buffer (pH 4.6)/ACN (85/15, v/v); UV detection wavelength, 230 nm; total flow rate: 400 nL/min; injection volume: 20 nL.

Table S1. Permeability of the Tei-ICNEML organic-silica hybrid monolith column.

Mobile phase	Relative polarity	Viscosity η ($\times 10^{-3}$ Pa·s)	Permeability K ($\times 10^{-14}$ m^2)
ACN/H_2O (50/50)	/	0.820	0.82
MeOH	0.762	0.544	1.76
ACN	0.460	0.369	1.81

Relative polarity and viscosity data of pure liquids were obtained from Ref. [8,22].

Table S2. Reproducibility of the Tei-ICNEML organic-silica hybrid monolith column.

	Average retention factor (RSD)		Average selectivity α (RSD)	Average resolution *Rs* (RSD)
	k_1	k_2		
Run to run (n=6)	1.20 (0.59%)	1.76 (0.63%)	1.47 (0.41%)	1.55 (0.72%)
Day to day (n=3)	1.21 (1.30%)	1.78 (1.52%)	1.47 (1.19%)	1.51 (2.01%)
Column to column (n=3)	1.24 (3.26%)	1.76 (3.97%)	1. 42 (2.11%)	1.50 (4.15%)

Experimental conditions: column dimensions: 10 cm × 100 μm I.D.; mobile phase: MeOH/ACN/TEA/HAc (80:20:0.03%:0.055%, v/v/v/v); total flow rate: 20 μL/min; UV detection wavelength: 225 nm; injection volume: 50 nL. Sample: Metoprolol.

Acknowledgements

M. L. Marina and E. Sánchez-López thank the Ministry of Economy and Competitiveness (Spain) for research project CTQ2013-48740-P and CTQ2016-76368-P. D. Xu and E. Sánchez-López thank the Comunidad of Madrid and European funding from FEDER program for their contracts (AVANSECAL-CM program, ref. S2013/ABI-3028). This work was also supported by the Science and Technology Planning Project of Guangdong Province, China (2016A040403056 and 2015A020211018) and the International Science & Technology Cooperation Program of Guangzhou, China (201807010022).

References

[1] T.J. Ward, K.D. Ward, Chiral separations: a review of current topics and trends, Anal. Chem., 84 (2011) 626-635.

[2] E. Eljarrat, P. Guerra, D. Barceló, Enantiomeric determination of chiral persistent organic pollutants and their metabolites, TrAC, 27 (2008) 847-861.

[3] J. Guo, Q. Zhang, Y. Peng, Z. Liu, L. Rao, T. He, J. Crommen, P. Sun, Z. Jiang, A facile and efficient one-step strategy for the preparation of t‐cyclodextrin monoliths, J. Sep. Sci., 36 (2013) 2441-2449.

[4] Q. Zhang, J. Guo, F. Wang, J. Crommen, Z. Jiang, Preparation of a β-cyclodextrin functionalized monolith *via* a novel and simple one-pot approach and application to enantioseparations, J. Chromatogr. A, 1325 (2014) 147-154.

[5] Y. Okamoto, T. Ikai, J. Shen, Controlled immobilization of polysaccharide derivatives for efficient chiral separation, Isr. J. Chem., 51 (2011) 1096-1106.

[6] F. Qin, C. Xie, S. Feng, J. Ou, L. Kong, M. Ye, H. Zou, Monolithic silica capillary column with coated cellulose tris (3,5-dimethylphenylcarbamate) for capillary electrochromatographic separation of enantiomers, Electrophoresis, 27 (2006) 1050-1059.

[7] A.P. Kumar, J.H. Park, Enantioseparation on cellulose dimethylphenylcarbamate-modified zirconia monolithic columns by reversed-phase capillary electrochromatography, J. Chromatogr. A, 1217 (2010) 4494-4500.

[8] D. Xu, H. Shao, R. Luo, Q. Wang, E. Sánchez-López, S. Fanali, M.L. Marina, Z. Jiang, A facile and efficient single-step approach for the fabrication of vancomycin functionalized polymer-based monolith as chiral stationary phase for nano-liquid

chromatography, J. Chromatogr. A, 1557 (2018) 43-50.
[9] G. Ding, A. Tang, Capillary electrochromatographic separation of racemates on a norvancomycin-bonded monolithic column under reversed-phase conditions, J. Chromatogr. A, 1208 (2008) 232-238.
[10] R. Mallik, T. Jiang, D.S. Hage, High-performance affinity monolith chromatography: development and evaluation of human serum albumin columns, Anal. Chem., 76 (2004) 7013-7022.
[11] T. Hong, Y. Zheng, W. Hu, Y. Ji, Preparation and evaluation of bovine serum albumin immobilized chiral monolithic column for affinity capillary electrochromatography, Anal. Biochem., 464 (2014) 43-50.
[12] M.G. Schmid, J. Koidl, P. Wank, G. Kargl, H. Zöhrer, G. Gübitz, Enantioseparation by ligand-exchange using particle-loaded monoliths: capillary-LC versus capillary electrochromatography, J. Biochem. Biophys. Methods, 70 (2007) 77-85.
[13] Z. Chen, T. Hobo, Chemically L-prolinamide modified monolithic silica column for enantiomeric separation of dansyl amino acids and hydroxy acids by capillary electrochromatography and μ‐high performance liquid chromatography, Electrophoresis, 22 (2001) 3339-3346.
[14] S. Mizrahi, D. Rizkov, A.I. Shames, O. Lev, Chiral separation of dansyl amino acids by ligand exchange capillary electrochromatography in a low molecular weight organogel, Electrophoresis, 29 (2008) 3941-3948.
[15] H.F. Wang, Y.Z. Zhu, X.P. Yan, R.Y. Gao, J.Y. Zheng, A room temperature ionic liquid (RTIL) mediated, non-hydrolytic sol-gel methodology to prepare molecularly imprinted, silica-based hybrid monoliths for chiral separation, Adv. Mater., 18 (2006) 3266-3270.
[16] H.Y. Zong, X. Liu, Z.S. Liu, Y.P. Huang, Molecular crowding-based imprinted monolithic column for capillary electrochromatography, Electrophoresis, 36 (2015) 818-824.
[17] M. Ruan, Q. Wang, H. Wu, Y. Wang, H. Han, Z. Jiang, Preparation and evaluation of tert-leucine derivative functionalized polymeric monoliths for micro-liquid chromatography, Electrophoresis, 38 (2017) 3020-3028.
[18] M.D. Slater, J.M. Fréchet, F. Svec, In‐column preparation of a brush-type chiral stationary phase using click chemistry and a silica monolith, J. Sep. Sci., 32 (2009) 21-28.

[19] M. Lämmerhofer, E.C. Peters, C. Yu, F. Svec, J.M. Fréchet, W. Lindner, Chiral monolithic columns for enantioselective capillary electrochromatography prepared by copolymerization of a monomer with quinidine functionality. 1. Optimization of polymerization conditions, porous properties, and chemistry of the stationary phase, Anal. Chem., 72 (2000) 4614-4622.
[20] D. Xu, Q. Wang, E. Sánchez-López, Z. Jiang, M.L. Marina, Preparation of an O-[2-(methacryloyloxy)-ethylcarbamoyl]-10, 11-dihydroquinidine-silica hybrid monolithic column for the enantioseparation of amino acids by nano-liquid chromatography, J. Chromatogr. A, 1593 (2019) 63-72.
[21] Q. Wang, E. Sánchez-López, H. Han, H. Wu, P. Zhu, J. Crommen, M.L. Marina, Z. Jiang, Separation of N-derivatized di-and tri-peptide stereoisomers by micro-liquid chromatography using a quinidine-based monolithic column-Analysis of L-carnosine in dietary supplements, J. Chromatogr. A, 1428 (2016) 176-184.
[22] Q. Wang, P. Zhu, M. Ruan, H. Wu, K. Peng, H. Han, G.W. Somsen, J. Crommen, Z. Jiang, Chiral separation of acidic compounds using an O-9-(tert-butylcarbamoyl) quinidine functionalized monolith in micro-liquid chromatography, J. Chromatogr. A, 1444 (2016) 64-73.
[23] S. Dixit, I.S. Lee, J.H. Park, Carbamoylated azithromycin incorporated zirconia hybrid monolith for enantioseparation of acidic chiral drugs using non-aqueous capillary electrochromatography, J. Chromatogr. A, 1507 (2017) 132-140.
[24] M. Kim, J.H. Park, Enantioseparation of chiral acids and bases on a clindamycin phosphate-modified zirconia monolith by capillary electrochromatography, J. Chromatogr. A, 1251 (2012) 244-248.
[25] X. Dong, J. Dong, J. Ou, Y. Zhu, H. Zou, Preparation and evaluation of a vancomycin-immobilized silica monolith as chiral stationary phase for CEC, Electrophoresis, 28 (2007) 2606-2612.
[26] M. Kele, G. Guiochon, Repeatability and reproducibility of retention data and band profiles on six batches of monolithic columns, J. Chromatogr. A, 960 (2002) 19-49.
[27] B. Chankvetadze, Monolithic chiral stationary phases for liquid-phase enantioseparation techniques, J. Sep. Sci., 33 (2010) 305-314.
[28] C. Gatschelhofer, M.G. Schmid, K. Schreiner, T.R. Pieber, F.M. Sinner, G. Gübitz, Enantioseparation of glycyl-dipeptides by CEC using particle-loaded monoliths prepared by ring-opening metathesis polymerization (ROMP), J. Biochem. Biophys. Methods, 69 (2006) 67-77.

[29] M.G. Schmid, J. Koidl, C. Freigassner, S. Tahedl, L. Wojcik, T. Beesley, D.W. Armstrong, G. Gübitz, New particle‐loaded monoliths for chiral capillary electrochromatographic separation, Electrophoresis, 25 (2004) 3195-3203.
[30] M. Kato, N. Matsumoto, K. Sakai-Kato, T. Toyo'oka, Investigation of chromatographic performances and binding characteristics of BSA-encapsulated capillary column prepared by the sol-gel method, J. Pharm. Biomed. Anal., 30 (2003) 1845-1850.
[31] K. Sakai-Kato, M. Kato, H. Nakakuki, T. Toyo'oka, Investigation of structure and enantioselectivity of BSA-encapsulated sol-gel columns prepared for capillary electrochromatography, J. Pharm. Biomed. Anal., 31 (2003) 299-309.
[32] S. Xu, R. Mo, C. Jin, X. Cui, R. Bai, Y. Ji, Mesoporous silica nanoparticles incorporated hybrid monolithic stationary phase immobilized with pepsin for enantioseparation by capillary electrochromatography, J. Pharm. Biomed. Anal., 140 (2017) 190-198.
[33] T. Zhu, K.H. Row, Preparation and applications of hybrid organic-inorganic monoliths: a review, J. Sep. Sci., 35 (2012) 1294-1302.
[34] J. Ou, Z. Liu, H. Wang, H. Lin, J. Dong, H. Zou, Recent development of hybrid organic‐silica monolithic columns in CEC and capillary LC, Electrophoresis, 36 (2015) 62-75.
[35] A. Berthod, Y. Liu, C. Bagwill, D.W. Armstrong, Facile liquid chromatographic enantioresolution of native amino acids and peptides using a teicoplanin chiral stationary phase, J. Chromatogr. A, 731 (1996) 123-137.
[36] D.W. Armstrong, Y. Liu, K.H. Ekborgott, A covalently bonded teicoplanin chiral stationary phase for HPLC enantioseparations, Chirality, 7 (1995) 474-497.
[37] M.P. Gasper, A. Berthod, U.B. Nair, D.W. Armstrong, Comparison and modeling study of vancomycin, ristocetin A, and teicoplanin for CE enantioseparations, Anal. Chem., 68 (1996) 2501-2514.
[38] E. Tesařová, Z. Bosáková, I. Zusková, Enantioseparation of selected N-tert butyloxycarbonyl amino acids in high-performance liquid chromatography and capillary electrophoresis with a teicoplanin chiral selector, J. Chromatogr. A, 879 (2000) 147-156.
[39] R. Luo, H. Han, J. Liu, D. Xu, Q. Wang, S. Fanali, Z. Jiang, Preparation and application of teicoplanin functionalized polymeric monolith for enantioseparation of

chiral drugs, J. Pharm. Biomed. Anal., Under review.
[40] H. Lin, J. Ou, Z. Zhang, J. Dong, M. Wu, H. Zou, Facile preparation of zwitterionic organic-silica hybrid monolithic capillary column with an improved “one-pot” approach for hydrophilic-interaction liquid chromatography (HILIC), Anal. Chem., 84 (2012) 2721-2728.
[41] E. Loeser, P. Drumm, Using strong injection solvents with 100% aqueous mobile phase in RP‐LC, J. Sep. Sci., 29 (2006) 2847-2852.
[42] J. Layne, T. Farcas, I. Rustamov, F. Ahmed, Volume-load capacity in fast-gradient liquid chromatography: Effect of sample solvent composition and injection volume on chromatographic performance, J. chromatogr. A, 913 (2001) 233-242.

III. 3.

Preparation of O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine-silica hybrid monolithic for nano-liquid chromatography

III.3. Preparation of O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine-silica hybrid monolithic for nano-liquid chromatography

III.3.1. Preface

Nowadays, many possibilities are available to carry out a successful chiral separation, playing micro-separative techniques a crucial role due to the advantages derived from their inherent low dimensions. Among the different miniaturized strategies to achieve chiral separations, the use of chiral columns in nano-LC or CEC has attracted much interest. Specifically, monolithic stationary phases functionalized with quinidine or quinine have been used in enantioseparation by these two techniques and have demonstrated to offer an excellent chiral separation ability for various kinds of acidic compounds. Recently, several quinine and quinidine-based monolithic columns were developed by Lämmerhofer et al. including the organic polymer-based monoliths, and silica-based monoliths. The aim of those works was mainly to describe the preparation strategy for those monolithic columns and their ability to enantioseparate several N-derivatized amino acids and 2-aryloxypropionic acids. In addition, Wang and co-workers developed several organic polymer-based quinidine monoliths to separate 44 N-derivatized amino acids and 53 small peptides. So far, the major category of quinidine functionalized monoliths is silica- or polymer-based.

Organic-inorganic hybrid monolithic columns have been developed because of their excellent permeability, higher pH stability, high surface, and superior performance. Compared with the polymer-based and silica-based monoliths, organic-silica hybrid monolith shows much less shrinkage. However, to the best of our knowledge, a quinidine functionalized hybrid monolith has never been reported before.

Regarding quinine functionalized hybrid monoliths, only a few have been reported so far being based on the so called multistep approaches. Nevertheless, the preparation processes are rather complicated, and included hydrolysis and filling the resulting polymerization mixture into the capillary for following polycondensation and polymerization. Given the complexity of the multistep procedure, a shift towards “one-step” strategies should be encouraged such as the one developed by Zou *et al.* [18, 23]. Taking this into account, it would seem appropriate to adapt the synthesis of quinidine or quinine functionalized hybrid monolith as a “one-pot” and this was the objective of the research work carried out in this PhD Thesis.

III.3.2. Objectives

The specific objectives of this work were:

- To synthesize the quinidine functional monomer for the monolithic column.
- To prepare novel quinidine functionalized silica-hybrid monolithic columns by a "one-step" strategy.
- To optimize the quinidine functionalized silica-hybrid monolithic columns to enhance the enantioresolution, enantioselectivity and column efficiency.
- To evaluate and characterize the chromatographic performance of the novel monolithic columns.
- To enantioseparate N-derivatized amino acids in the different separation modes.
- To compare the chiral recognition ability under the reversed phase mode and organic phase mode for N-derivatized amino acids.

III.3.3. Results

The results obtained in this work are included in the following scientific article:

Article 3: *Preparation of an O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine-silica hybrid monolithic column for the enantioseparation of amino acids by nano-liquid chromatography*

D. Xu, Q. Wang, E. Sánchez-López, Z. Jiang, M. L. Marina

J. Chromatogr. A, 2019, 1593, 63-72.

Article 3

Preparation of an O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11- dihydroquinidine silica hybrid monolithic column for the enantioseparation of amino acids by nano-liquid chromatography

D. Xu, Q. Wang, E. Sánchez-López, Z. Jiang, M. L. Marina

J. Chromatogr. A, 2019, 1593, 63-72.

Abstract

An O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine (MQD)-silica hybrid monolithic column was prepared by a facile “one-step” strategy within a 100 μm I.D. capillary. The influence of the methanol, ethylene glycol and water volume ratio, reaction temperature and time, cetyltrimethylammonium bromide and MQD monomers content and volume ratio of tetramethoxysilane and vinyltrimethoxysilane was investigated to obtain a satisfactory morphology of monolithic columns. The optimized MQD-silica hybrid monolithic column was evaluated in terms of permeability, stability, efficiency, reproducibility, and was characterized by scanning electron microscopy and nano-liquid chromatography. Among the 52 N-derivatized protein and non-protein amino acids, a total of 44 analytes could be baseline enantioseparated using the optimized conditions in either reversed phase mode (RPM) or polar organic phase mode (POM). The results showed that POM (ACN/MeOH/HAc/TEA (60/40/0.055/0.005, v/v/v/v)) offered better performance than RPM (10 mM ammonium acetate/ACN (30/70, v/v) (apparent pH=5.3)) in terms of enantioresolution and efficiency with shorter analysis times.

Keywords:

Quinidine, Enantioseparation, Hybrid monolith, Nano-Liquid Chromatography

1. Introduction

Chiral analysis is a relevant topic in separation sciences due to its profound impact in different fields such as pharmaceutical, agrochemical and food industry. Nowadays, many possibilities are available to carry out a successful chiral separation, in which micro-separative techniques play a vital role due to the advantages derived from their inherent low dimensions. Among the different miniaturized strategies to achieve chiral separations, the use of chiral columns in nano-liquid chromatography (nano-LC) or capillary electrochromatography (CEC) has attracted much interest. Specifically, monolithic stationary phases functionalized with quinidine or quinine have been used in enantioseparation by these two techniques [1-3] and have demonstrated to offer an excellent chiral separation ability for various kinds of acidic compounds such as amino acids [4-5], small peptides [6], or profens [7-8]. Recently, several quinine and quinidine-based monolithic columns have been developed by Lämmerhofer *et al.*, such as organic polymer-based monoliths, O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine-*co*-2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate [1, 9-10], O-9-(*tert*-butylcarbamoyl)-11-[2-(methacryloyloxy)ethylthio]-10,11-dihydroquinine-*co*-2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate [2], O-9-(*tert*-butylcarbamoyl)-quinine-*co*-glycidylmethacrylate-*co*-ethylene dimethacrylate [11], O-9-(*tert*-butylcarbamoyl)-quinine-*co*-3-mercaptopropyl methylsiloxane-*co*-glycidylmethacrylate-*co*-ethylene dimethacrylate [12-13]; and silica-based monoliths, O-9-(*tert*-butylcarbamoyl)-11-[2-(methacryloyloxy)ethylthio]-10,11-dihydroquinine [14] and O-9-(*tert*-butylcarbamoyl)-quinine [15]. The aim of those works was mainly to describe the preparation strategy for those monolithic columns and their ability to enantioseparate several N-derivatized amino acids and 2-aryloxypropionic acids. Wang and co-workers developed several organic polymer-based quinidine monoliths, namely O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine-*co*-2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate (poly(MQD-*co*-HEMA-*co*-EDMA)) [4, 16], and O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine-*co*-ethylene dimethacrylate (poly (MQD-*co*-EDMA)) [5], and O-9-(*tert*-butylcarbamoyl)-11-[2-(methacryloyloxy)ethylthio]-10,11-dihydroquinidine (poly (MBQD-*co*-HEMA-EDMS)) [3]. On the optimized monolithic columns, 44 N-derivatized amino acids and 53 small peptides were enantioseparated [4, 6]. So far, the major category of quinidine functionalized monoliths is silica- or polymer-based.

Organic-inorganic hybrid monolithic columns have been developed because of their excellent permeability, higher pH stability, high surface, and superior performance [17-19]. Compared with the polymer-based and silica-based monoliths, organic-silica hybrid monolith shows much less shrinkage [20-22]. However, to the best of our knowledge, a quinidine functionalized hybrid monolith has never been reported before. Regarding quinine functionalized hybrid monoliths, only a few have been reported so far being based on so called multistep approaches [7, 8]. Tran *et al.* prepared a quinine functionalized silica/zirconia hybrid monolith through a convention sol-gel process of 3-triethoxysilypropylcarbamated quinine and zirconium tetrabutoxide in solution containing polyethylene glycol (PEG), acetic acid, *n*-butanol and water [7]. The obtained hybrid monolith exhibited good enantioselectivity for sever profens and dinitrobenzoyl (DNB) amino acids in CEC mode. In another work, they also prepared quinine functionalized silica hybrid monolith *via* both sol-gel chemistry and free radical polymerization of *tert*-butylcarbamoylquinine (*t*BuCQUI) vinyltrimethoxysilane (VTMS) and tetramethoxysilane (TMOS) [8]. Nevertheless, the preparation processes is rather complicated, where *t*BuCQUI was added after hydrolyzation (at 0 ºC for 1 h), and then the resulting polymerization mixture was filled in to capillary for following polycondensation (at 30 ºC for 10 h) and polymerization (at 120 ºC for 10 h). Given the complexity of the multistep procedure a shift towards "one-step" strategies should be encouraged such as the one developed by Zou *et al.* [18, 23]. Therein, authors developed a straightforward "one-pot" approach for the preparation of perphenylcarbamoylated β-cyclodextrin and phenyl-silica hybrid monolith. By adding cetyltrimethylammonium bromide (CTAB), MeOH and DMF, a homogeneous solution of all reagents (including hydrophobic organic monomer and hydrophilic methoxysilanes) can be easily obtained at room temperature. The resulting polymerization mixture was filled into capillary for following in-site hydrolyzation, polycondensation and polymerization. The proposed approach not only provided a solution for preparing organic-inorganic hybrid monoliths with hydrophobic monomers, but also significantly simplified the preparation process. Taking this into account, it would seem appropriate to adapt the synthesis of quinidine or quinine functionalized hybrid monolith as a "one-pot".

In this work, an O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine (MQD)-silica hybrid monolithic column was prepared within a 100 μm I.D. capillary through a facile "one-step" strategy. In order to obtain a satisfactory permeability, the methanol (MeOH)/ethylene glycol (EG) (v/v), H_2O/ammonium hydroxide ($NH_3 \cdot H_2O$)

(v/v), CTAB (mg), TMOS/VTMS (v/v), monomer (MQD) (mg) in the pre-polymerizable mixture and the reaction temperature and time were optimized in order to obtain the best column efficiency and enantioresolution for a set of N-derivatized amino acids. The composition of mobile phase was evaluated on the optimized MQD-silica hybrid monolithic column under the RPM and POM conditions, and a total of 52 N-derivatized protein and non-protein amino acids were enantioseparated under both mobile phase modes. Finally, the two different mobile phase modes were compared in terms of enantioselectivity (α), analysis time, enantioresolution (Rs) and column efficiency (N).

2. Materials and methods

2.1. Reagents and solvents

10,11-dihydroquinidine, 2,2'-azobisisobutyronitrile (AIBN), 3-(trimethoxysilyl)-propylmethacrylate (γ-MAPS), 2-isocyanatoethyl methacrylate (ICNEML), VTMS, CTAB, TMOS and EG were acquired from Aladdin Chemicals (Shanghai, China). MeOH, tetrahydrofuran (THF), chloroform ($CHCl_3$), acetonitrile (ACN) and acetic acid (HAc) were acquired from Scharlau Chemie (Barcelona, Spain). Triethylamine (TEA) and 9-fluorenylmethoxycarbonyl (FMOC) chloride were obtained from Fluka (Buchs, Switzerland). $NH_3 \cdot H_2O$, boric acid (H_3BO_3) and pentane were from Sigma (St. Louis, Missouri, USA), and ammonium acetate was from Merck (Darmstadt, Germany). 3,5-dinitrobenzoyl (3,5-DNB) chloride, *p*-nitrobenzoyl (*p*-NB) chloride, 3,5-dimethoxybenzoyl (3,5-DMB) chloride, 3,5-dichlorobenzoyl (3,5-DCIB) chloride, *m*-chlorobenzoyl (*m*-CIB) chloride, *p*-chlorobenzoyl (*p*-CIB) chloride, benzoyl (B) chloride and propylene oxide were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). DL-serine, DL-threonine, DL-cysteine, DL-alanine, DL-valine, DL-leucine, DL-methionine, DL-phenylalanine, DL-tryptophan, and DL-citrulline standards were obtained from Fluka (Buchs, Switzerland), while DL-isoleucine, DL-norvaline, DL-norleucine, and DL-methionine sulfone were obtained from Sigma (St. Louis, Missouri, USA). All the N-derivatized amino acids were synthesized as previously described [4].

Fused-silica capillaries (375 μm O.D. × 100 μm I.D.) were obtained from Ruifeng Chromatography Ltd. (Hebei, China). Distilled water was purified using a Milli-Q water system from Millipore (Massachusetts, USA). Reversed phase mobile phases

were prepared by mixing ACN and ammonium acetate buffer solvents and adjusting the apparent pH to the desired value using HAc. Polar organic mobile phases were set up by mixing the desired ratio of ACN and MeOH, and then adding various amounts of HAc and TEA. All mobile phases were subjected to filtration through a 0.22 μm membrane and sonicated prior use.

2.2. Instrumentation

The molecular mass of the MQD monomer was determined on an AB Sciex QTrap 4500 mass spectrometer (California, USA). A Jinghong DKS22 water bath (Shanghai, China) was used for thermally initiated copolymerization. Scanning electron microscopy (SEM) experiments were performed on a Zeiss Gemini ultra-55 SEM (Deutschland, Germany) at an acceleration voltage of 5 kV. All nano-LC experiments were conducted on a laboratory self-assembled instrument. The system consisted of a Shimadzu LC-20AD pump (Kyoto, Japan), a Linear Instruments UVIS-200 detector (California, USA), and a Valco four-port injection valve with a 20 nL internal loop (Houston, USA). In order to reduce the flow rate, a stainless-steel tee (Cheminert, Valco Instruments Houston, Texas, USA) with a flow split capillary (150 mm × 25 μm I.D.) was employed before the injection valve. Data acquisition and handling were performed using the software Chromatostation N200 (Zhejiang University, China). All chromatograms were converted to a text file and then redrawn using Microcal Origin 8.5. The pH values of buffer solutions were measured by a 744 pH meter (Herisau, Switzerland).

2.3. Preparation of the MQD-silica hybrid monolithic column

At the beginning, the fused-silica capillary inner wall was modified using γ-MAPS/MeOH (50/50, v/v), in order to provide the anchoring sites for the bulk polymer [3], and the chiral functional monomer MQD was synthesized according to a previously reported method [1, 4], the MS spectrum (ESI+) of the purified product is depicted in **Fig. S1** where an ion at *m/z* 482.2, i.e. the $[M+H]^+$ ion from the functional MQD monomer, is observed.

The schematic representation of the preparation of the MQD-silica hybrid monolithic column is illustrated in **Fig. 1**. The pre-polymerizable mixture for preparing the MQD-silica hybrid monolithic column was obtained by mixing the MQD (6.0 mg), MeOH

(100 μL), EG (30 μL), CTAB (1.6 mg), H_2O (30 μL), $NH_3 \cdot H_2O$ (0.02 M, 30 μL), TMOS (60 μL), VTMS (80 μL), and AIBN (1 mg) in a 2-mL vial. After sonicating for 5 min at room temperature, a homogeneous solution was obtained and was then introduced into the 30 cm pretreated capillary. Then, both ends of the capillaries were sealed with GC septa and submerged into the water bath (40 °C) for 12 h and followed by another 12 h at 60 °C. The unreacted CTAB and other residuals were removed by flushing the column with methanol. The obtained monolith column was cut to 15 cm for nano-LC analysis. A 2-5 mm length of the monolith column was used for SEM analysis (**Fig. S2**).

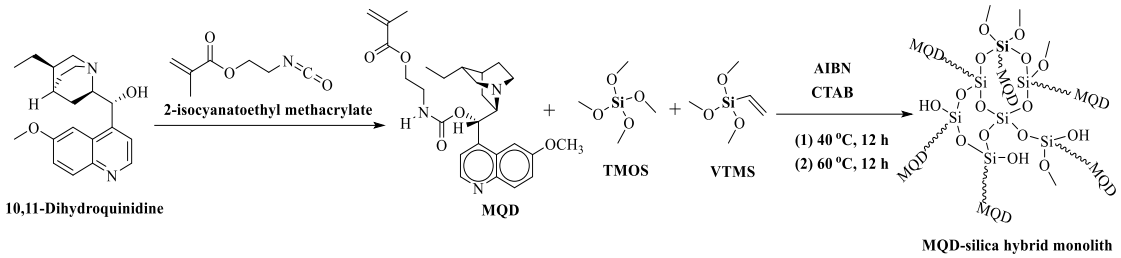


Fig. 1. Schematic representation of the preparation of the MQD-silica hybrid monolith

3. Results and discussion

3.1. Preparation and characterization of the MQD-silica hybrid monolithic column

3.1.1. Preparation of the MQD-silica hybrid monolithic column

Previous research has proved that the physicochemical and chromatographic properties of the hybrid monolithic column can be controlled by changing the pre-polymerizable mixture [18]. In order to obtain a hybrid monolithic column with a good permeability, the solvent combination (MeOH, EG, H_2O and $NH_3 \cdot H_2O$), the content of the CTAB, MQD monomer, the ratio of TMOS/VTMS (v/v) and the reaction time and temperature were investigated under the microscope, as shown in **Table 1**.

First, the combination of different solvents (H_2O, MeOH, ethanol, DMF, DMSO, THF, benzyl alcohol, EG, ethyl acetate, 1,4-butanediol and 1-propanol) was tested. Among the different solvents evaluated, a ternary system containing H_2O, MeOH and EG was selected because of its good solubility for MQD. Then, the influence of the MeOH/EG ratio (v/v) and the content of H_2O was studied by varying them from 95/35 (column **C1**) to 105/25 (column **C3**), and from 25 μL (column **C4**) to 35 μL (column **C5**), respectively. As can be observed in the microscope images from **Table 1**, the column **C2** prepared with MeOH/EG/H_2O/$NH_3 \cdot H_2O$ (100/30/30/30, v/v/v/v) exhibited

a very good morphology in the microscope images when compared to columns **C1** (slack morphology), **C3** (semitransparent morphology), **C4** (transparent morphology) and **C5** (slack morphology), therefore, it was selected for the following studies.

The CTAB content has proved to have a strong impact on the hybrid column morphology [18]. Different weight fractions of CTAB were assayed: 1.2 mg (column **C6**), 1.6 mg (column **C2**) and 2.0 mg (column **C7**). The column **C6** showed a semitransparent morphology because of the low content of CTAB, but the column **C7** was slack. Because CTAB acts as a supramolecular template in the reaction process for hybrid monolithic column, high amounts of CTAB in the pre-polymerizable mixture results in a slack morphology for the monolith. Therefore, an amount of 1.6 mg of CTAB (column **C2**) was selected for the next studies.

In order to get a homogeneous solution which can be introduced into the capillary, the reaction of hydrolysis and polycondensation necessary to prepare the hybrid monolithic column was adjusted based on the ratio of TMOS/VTMS and the content of MQD. Different TMOS/VTMS ratios (μL, v/v) were tested from 55:85 (column **C8**) to 65:75 (column **C9**) and the content of MQD was tested from 4.0 mg (**C10**) to 8.0 mg (**C11**). All the morphologies obtained can be seen in **Table 1**. When decreasing the content of VTMS and MQD, while other conditions were kept constant, the morphology image of the monolithic column became slacker. Finally, a TMOS/VTMS ratio of 60:80 (μL, v/v) and MQD 6.0 mg (column **C2**) was chosen for the following experiment because it was a transparent and homogeneous solution which was easier to introduce into the capillary.

Another aspect to take into account is the reaction temperature and time as they affect the morphology and permeability of the monolithic column. Three different conditions were tested: 40 ºC during 12 h followed by 60 ºC during 12 h (column **C2**), 40 ºC for 24 h (column **C12**) and 60 ºC during 24 h (column **C13**). The microscope images of the columns prepared under the different temperatures and times are shown in **Table 1**. Column **C2** was selected because of the monolithic column exhibited a good morphology image.

Based on these optimization experiments, the pre-polymerizable mixture consisting on MeOH (100 μL), EG (30 μL), H_2O (30 μL), $NH_3 \cdot H_2O$ (0.02 M, 30 μL), TMOS (60 μL), VTMS (80 μL), CTAB (1.6 mg), MQD (6.0 mg) and AIBN (1.0 mg) was chosen, conducting the reaction at 40 ºC for 12 h followed by 60 ºC for 12 h. As shown in **Fig. S2**, the morphology of the MQD-silica hybrid monolithic column (column **C2**) was

evaluated by SEM, and the SEM images indicated that the column **C2** had the morphology of a continuous skeleton and large through-pores, and the monolithic rod was tightly anchored on the inner wall of the capillary column.

Table 1. Effect of the preparation parameters for the MQD-silica hybrid monolithic columns.

Column	MeOH/EG (μL, v/v)	H_2O/ $NH_3 \cdot H_2O$ (μL, v/v)	CTAB (mg)	TMOS/VTMS (μL, v/v)	MQD (mg)	Temperature and Time (°C, h)	Morphology
C1	95/35	30/30	1.6	60/80	6.0	40 °C, 12 h; 60 °C, 12 h	
C2	100/30	30/30	1.6	60/80	6.0	40 °C, 12 h; 60 °C, 12 h	
C3	105/25	30/30	1.6	60/80	6.0	40 °C, 12 h; 60 °C, 12 h	
C4	100/30	25/30	1.6	60/80	6.0	40 °C, 12 h; 60 °C, 12 h	
C5	100/30	35/30	1.6	60/80	6.0	40 °C, 12 h; 60 °C, 12 h	
C6	100/30	30/30	1.2	60/80	6.0	40 °C, 12 h; 60 °C, 12 h	
C7	100/30	30/30	2.0	60/80	6.0	40 °C, 12 h; 60 °C, 12 h	
C8	100/30	25/30	1.6	55/85	6.0	40 °C, 12 h; 60 °C, 12 h	
C9	100/30	35/30	1.6	65/75	6.0	40 °C, 12 h; 60 °C, 12 h	
C10	100/30	30/30	1.6	60/80	4.0	40 °C, 12 h; 60 °C, 12 h	
C11	100/30	30/30	1.6	60/80	8.0	40 °C, 12 h; 60 °C, 12 h	
C12	100/30	30/30	1.6	60/80	6.0	40 °C, 24 h	
C13	100/30	30/30	1.6	60/80	6.0	60 °C, 24 h	

3.1.2. Physicochemical evaluation of the MQD-silica hybrid monolithic column

The permeability (*K*) of the monolithic column can be calculated using the following equation [24, 25]:

$$K = \frac{u\eta L}{\Delta P}$$

being *u* the linear velocity of the mobile phase, L the length of the column, ΔP the pressure drop across the column, and η the dynamic viscosity of the eluent. Toluene (ACN or MeOH as mobile phase) and thiourea (H_2O/ACN (50/50, v/v) as mobile phase) were selected as the dead time markers. As can be seen in **Table S1**, the calculated *K* values for the chosen column (column **C2**) were 2.95×10^{-14}, 2.93×10^{-14} and 2.47×10^{-14} m^2 when using ACN, MeOH and H_2O/ACN (50/50, v/v) as the mobile phases, respectively. As can be seen in **Fig. S3**, good mechanical stability was evidenced by good linearity between the linear velocity and the backpressure, in the range of 5-100 bar, the R^2 values for ACN, MeOH and H_2O/ACN (50/50, v/v) were 0.9996, 0.9997, 0.9988, respectively. Overall, the results indicated good permeability and mechanical stability for the optimized MQD-silica hybrid monolithic column in the solvents with different polarities. The Van Deemter plots for the MQD-silica hybrid monolithic column were also determined in both POM (100% ACN, toluene as the test analyte) and RPM (30% water/70% ACN, thiourea as the test analyte) modes. At the optimum linear velocity, the theoretical plate height values are 42.5 μm and 44.2 μm for POM and RPM, respectively.

3.1.3. Reproducibility of the MQD-silica hybrid monolithic column

The reproducibility of the MQD-silica hybrid monolithic column was evaluated through the RSD values for retention factors (k_1, k_2), α and *Rs* of the N-derivatized racemic *p*-NB-leucine using a mixture of 10 mM ammonium acetate/ACN (30/70, v/v) (apparent pH 5.3) as mobile phase (see **Table S2**). The run-to-run RSDs (n=6) for k_1 and k_2 were 1.81% and 1.24%, while the column-to-column RSDs (n=5) for the retention factors were 2.53% and 3.05%, respectively. The batch-to-batch repeatability (n=3) for k_1 and k_2 was also adequate with RSDs values of 4.29% and 3.59%, respectively, in addition to day-to-day repeatability (n=3) which were 1.73% and 1.69%, respectively. RSD values of α and *Rs* were also satisfactory: ≤ 4.04. These data clearly indicated that the MQD-silica hybrid monolithic column has a satisfactory

reproducibility for enantioseparation in nano-LC.

3.2. Effects of the mobile phase composition

3.2.1. Reversed phase mode

As reported in previous studies [4], two N-derivatized amino acids (*p*-NB-leucine (see **Fig. 2**), 3,5-DMB-leucine (see **Table S3**)) were selected as test analytes, and the *k*, α and *Rs* were used to evaluate the MQD-silica hybrid monolithic column, in the RPM. In order to obtain satisfactory enantioseparation conditions, the concentration of the organic solvent, apparent pH and buffer concentration in the mobile phase were optimized.

The effect of the apparent pH of the mobile phase was evaluated from 4.3 to 6.3, while the mobile phase composition was kept constant (5 mM ammonium acetate/ACN (20/80, v/v)). As shown in **Fig. 2a** and **b**, the k_1 increased with increasing the apparent pH. On the other hand, *Rs* improved with increasing the apparent pH from 4.3 to 5.3, and then levelled off from 5.3 to 6.3. These results are also in agreement with previous studies [4]. A high apparent pH of the mobile phase would lead to a higher negative charge of the two N-derivatized amino acids (*p*-NB-leucine and 3,5-DMB-leucine), resulting in a stronger electrostatic interaction with the positively charged quinidine chiral stationary phase. Hence, an apparent pH of 5.3 was chosen for the following experiments.

The influence of the ACN concentration in the mobile phase on the k_1 and *Rs* also evaluated. As shown in **Fig. 2c**, both k_1 and *Rs* decreased when increasing the ACN concentration from 60 to 90% (v/v). This is due to the fact that a higher ACN concentration would cause a weaker hydrophobic interaction between the CSP and the tested enantiomers. Thus, 70% (v/v) ACN was selected for the following experiments as a compromise between the analysis time and enantioresolution.

The concentration of the ammonium acetate buffer was also studied from 1 to 30 mM. As shown in **Fig. 2d**, when increasing the buffer concentration in the mobile phase, both k_1 and *Rs* decreased. Moreover, it was found that when the concentration of buffer was low (1 mM), *Rs* were adequate for the two test analytes, while their analysis times were rather long (> 60 min). Finally, 10 mM ammonium acetate buffer was selected as the optimum mobile phase modifier.

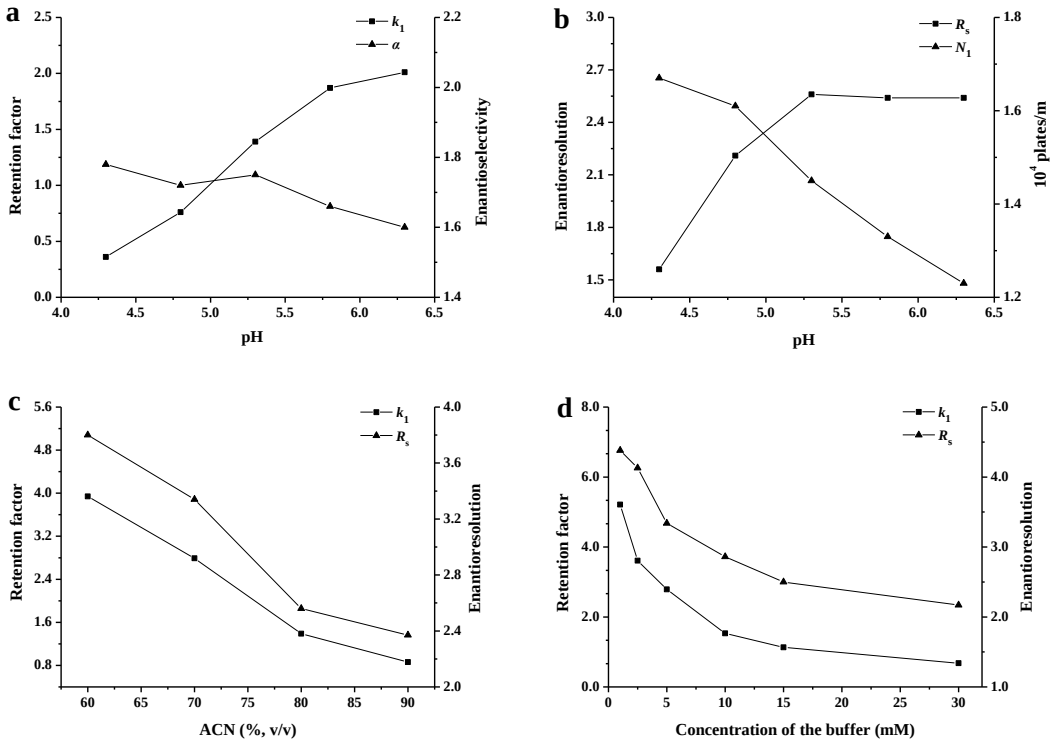


Fig. 2. Effect of the apparent pH values on (a) retention factor and enantioselectivity; (b) enantioseparation and column efficiency; effect of the (c) content of ACN and (d) concentration of the buffer for *p*-NB-leucine on the retention factor and enantioresolution, in the reversed phase mode. Experimental conditions: (a) and (b) 5 mM ammonium acetate/ACN (20/80, v/v) (at the desired apparent pH values); (c) 5 mM ammonium acetate/ACN (at the desired ratio of ACN and buffer, v/v) (apparent pH=5.3); (d) ammonium acetate/ACN (at the desired concentration of the buffer) (30/70, v/v) (apparent pH=5.3). Column dimensions: 15 cm × 100 μm I.D.; UV detection wavelength: 254 nm; flow rate: 10 μL/min; injection volume: 20 nL.

Under the optimal RPM conditions which consist of 10 mM ammonium acetate/ACN (30/70, v/v) (apparent pH=5.3), 52 N-derivatized amino acids were tested. As shown in **Table 2**, 42 out of 52 were baseline enantioseparated (*Rs* > 1.5) on the monolithic column. However, most of N-derivatized FMOC amino acids were not baseline enantioseparated, except the FMOC-isoleucine and FMOC-valine (*Rs* were 1.55 and 1.71, respectively). **Fig. 3** displays the enantioseparation for *p*-NB-leucine, *p*-CIB-Alanine, *p*-CIB-Methionine, 3,5-DMB-Leucine, 3,5-DCIB-Alanine and *p*-NB-Methionine.

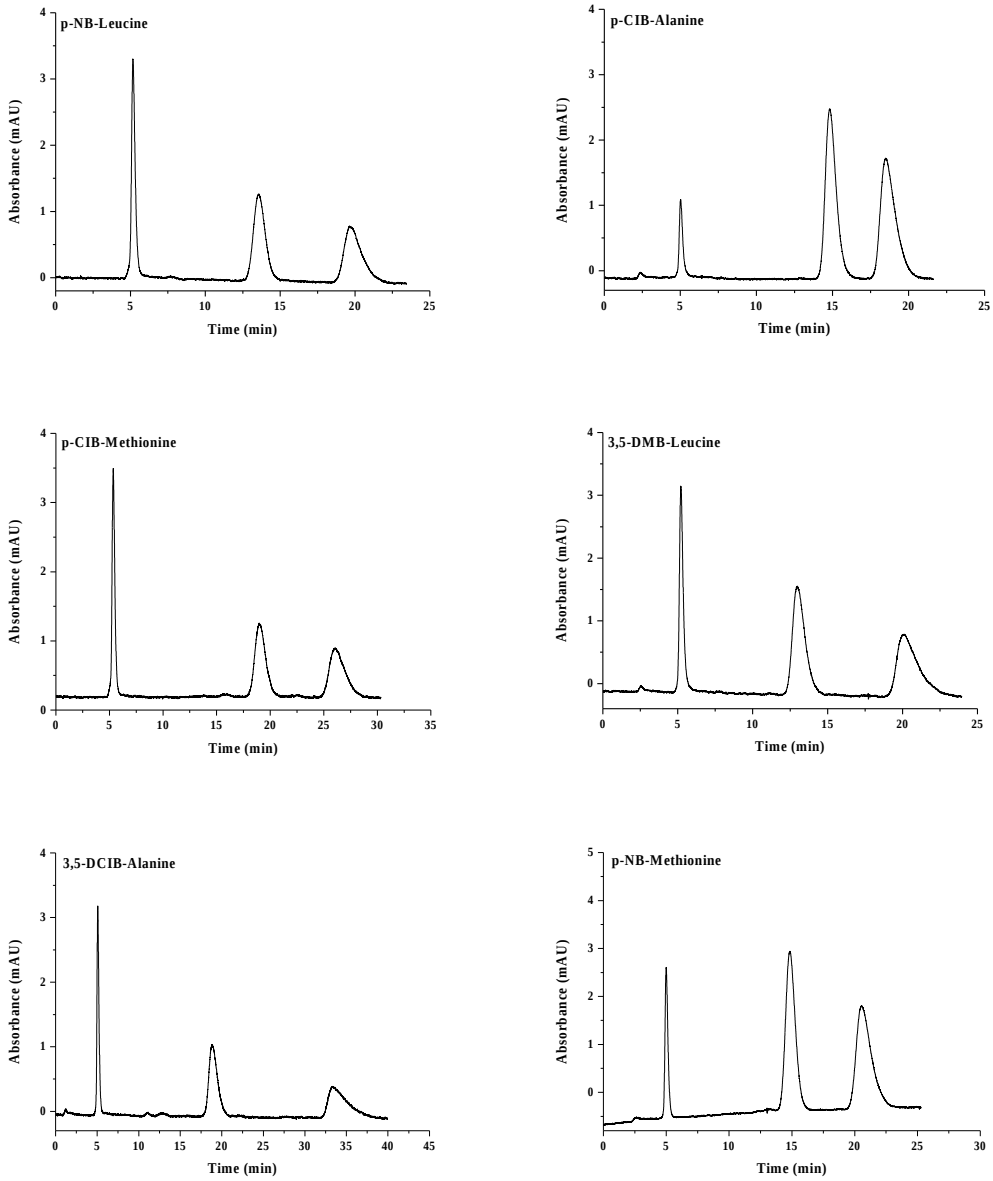


Fig. 3. Enantioseparation of some N-derivatized amino acids in the reversed phase mode. Mobile phase: 10 mM ammonium acetate/ACN (30/70, v/v) (apparent pH=5.3); Other experimental conditions are the same as in **Fig. 2**.

Table 2. Enantioseparation of *N*-derivatized amino acids in the reversed phase mode.

Sample	k_1	k_2	α	Rs	N_1 (m)	N_2 (m)
3,5-DNB-isoleucine	2.01	10.95	5.45	8.40	17300	12400
3,5-DNB-leucine	1.86	7.84	4.22	7.62	14500	11500
3,5-DNB-valine	1.85	8.31	4.49	10.10	14000	9200
3,5-DNB-norvaline	1.95	7.39	3.79	8.94	15100	10000

3,5-DNB-norleucine	2.19	8.31	3.79	8.97	14800	9500
3,5-DNB-methionine	2.24	7.80	3.48	8.37	14500	9500
3,5-DNB-tryptophan	3.30	12.83	3.89	8.47	14300	10100
3,5-DNB-citrulline	1.13	3.11	2.75	3.81	10100	7000
3,5-DNB-methionine sulfone	1.35	4.04	2.99	4.51	14000	6300
3,5-DNB-phenylalanine	2.79	10.77	3.86	6.83	16400	8700
3,5-DNB-alanine	1.50	2.45	1.63	2.65	17600	14600
3,5-DCIB-threonine	2.43	5.63	2.32	5.44	17100	15700
3,5-DCIB-norvaline	3.05	7.69	2.52	6.19	18300	13600
3,5-DCIB-valine	3.07	8.73	2.84	6.02	14700	9300
3,5-DCIB-serine	2.46	5.11	2.08	4.86	17700	16100
3,5-DCIB-alanine	2.71	5.58	2.06	4.33	16000	13500
3,5-DCIB-isoleucine	3.24	10.00	3.09	5.73	13200	9800
3,5-DCIB-norleucine	3.55	9.09	2.56	6.23	14200	11200
3,5-DCIB-methionine	3.54	8.60	2.43	5.61	15500	13800
3,5-DCIB-phenylalanine	4.43	10.99	2.48	6.13	15500	11200
3,5-DCIB-leucine	2.91	7.63	2.62	5.52	13500	10500
3,5-DCIB-cysteine	4.47	10.10	2.26	4.63	13800	9000
3,5-DCIB-tryptophan	4.66	10.86	2.33	4.78	11600	9700
3,5-DCIB-methionine sulfone	2.04	4.20	2.06	2.90	6400	5200
3,5-DCIB-citrulline	1.65	3.50	2.12	3.67	10900	8000
p-NB-leucine	1.53	2.65	1.73	2.86	15000	13600
p-NB-methionine	1.99	3.17	1.59	2.91	17000	15000
p-NB-alanine	1.50	2.14	1.43	2.17	20800	18700

3,5-DMB-alanine	1.47	2.41	1.64	2.69	18000	15700
3,5-DMB-methionine	1.91	3.35	1.75	2.87	14500	10200
3,5-DMB-threonine	1.39	2.27	1.63	2.54	18300	15000
3,5-DMB-leucine	1.37	2.62	1.91	3.11	13500	10900
3,5-DMB-norvaline	1.68	3.15	1.88	3.54	16400	14800
3,5-DMB-norleucine	1.93	3.60	1.87	3.81	16500	15000
m-CIB-methionine	2.73	4.57	1.67	3.47	16800	15200
m-CIB-alanine	2.12	3.20	1.51	2.62	18600	17600
m-CIB-leucine	2.21	3.97	1.80	3.39	13700	12200
m-CIB-threonine	1.88	3.01	1.60	2.85	16500	11000
p-CIB-leucine	2.08	3.28	1.58	2.42	13500	12800
p-CIB-methionine	2.55	3.87	1.52	2.65	16700	15000
p-CIB-alanine	1.95	2.68	1.37	2.04	20500	17600
B-methionine	2.19	2.87	1.31	1.66	16000	15500
B-threonine	1.49	1.93	1.29	1.53	19000	18200
B-leucine	1.60	2.13	1.33	1.51	13000	9500
FMOC-alanine	2.27	2.62	1.15	1.25	20300	18600
FMOC-tryptophan	4.24	5.02	1.18	1.26	13500	13000
FMOC-methionine	3.42	4.06	1.19	0.84	13200	8900
FMOC-valine	2.57	3.35	1.30	1.55	12000	9700
FMOC-norvaline	2.56	3.14	1.23	0.88	11000	8200
FMOC-norleucine	2.96	3.49	1.18	0.81	12300	9300
FMOC-methionine sulfone	1.91	2.33	1.22	0.81	13400	8500
FMOC-isoleucine	2.68	3.59	1.34	1.71	13900	8700

Experimental conditions are the same as in **Fig. 3**.

3.2.2. Polar organic phase mode

The second mode to be assayed was the POM. According to previous studies [6], N-derivatized amino acids can be enantioresolved in POM, specifically in a mobile phase consisting of ACN/MeOH and HAc/TEA. In this study, B-leucine (see **Fig. 4**) and 3,5-DCIB-leucine (see **Table S4**) were selected as the test analytes. The ratio of MeOH/ACN, HAc/TEA and the total concentration of HAc and TEA were optimized as in previous reports [26-28].

The influence of the MeOH/ACN ratio on the k_1 and *Rs* for the two test analytes was evaluated from 60/40 (%, v/v) to 20/80 (%, v/v), while the total concentration of TEA and HAc was kept constant. As shown in **Fig. 4a** and **b**, the k_1 increased gradually with decreasing the MeOH concentration from 60 to 20% (v/v). However, the *Rs* increased when increasing the content of MeOH from 20 to 40% (v/v), then decreased when further increasing the content to 60% (v/v). Hence, 40:60 (MeOH/ACN, v/v) was selected as the optimum mobile phase for the following experiments.

After, the effect of the total concentration of HAc+TEA was evaluated from 0.015 to 0.48% (v/v), while the ratio of HAc/TEA (5/1, v/v) was kept constant (see **Fig. 4c**). The N_1 increased when the total concentration of HAc+TEA increased from 0.015 to 0.48% (v/v). The highest total concentration of HAc+TEA would lead to higher efficiency and shorter analysis time, because of the weaker interaction between the enantiomers and the CSP when increasing the concentration of the competing acetate anion in the solvent. On the other hand, the *Rs* increased when increasing the concentration of HAc+TEA from 0.015 to 0.06% (v/v), then it decreased when the concentration of HAc+TEA increased up to 0.48% (v/v). Thus, a total concentration of 0.06% (v/v) for HAc+TEA was used for further experiments because it enabled to obtain the highest enantioresolution.

Finally, the HAc/TEA ratio was investigated. As can be seen in **Fig. 4d**, the *Rs* and N_1 of B-leucine increased when increasing the ratio of HAc/TEA from 3:1 (%, v/v) to 5:1 (%, v/v), then remained almost constant from 5:1 (%, v/v) to 29:1 (%, v/v). In the case of 3,5-DCIB-Leucine (see **Table S4**) the *Rs* kept increasing and remained almost constant at 11:1 (%, v/v) and 29:1 (%, v/v). As a compromise, a HAc/TEA ratio of 11:1 (%, v/v) was selected.

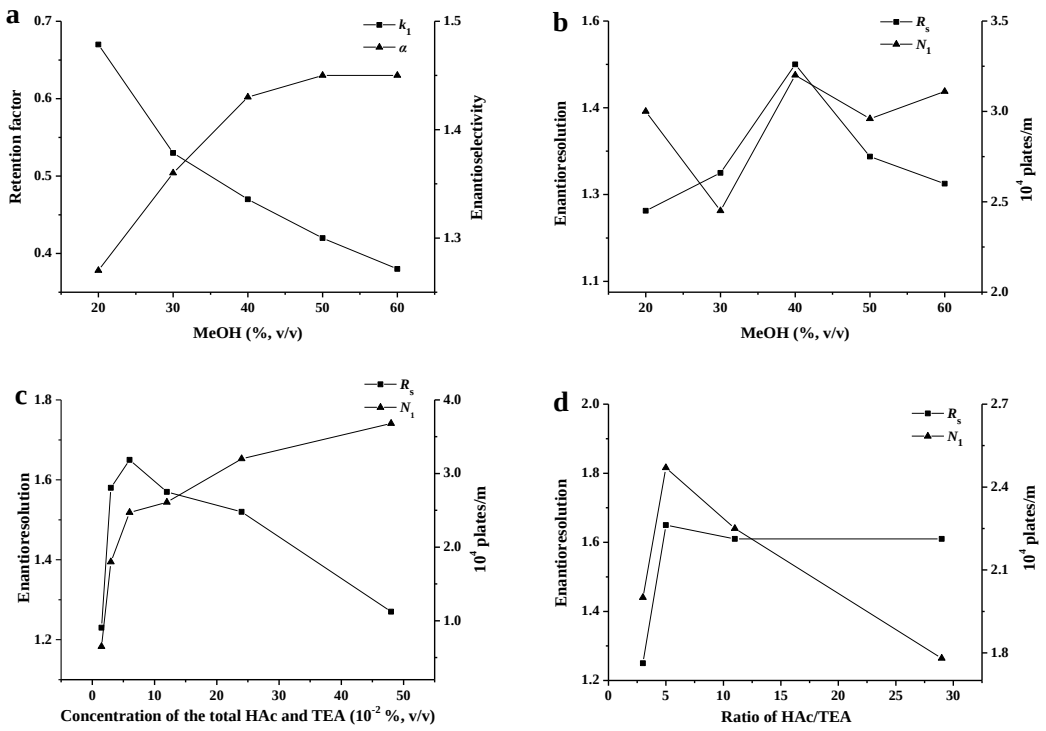


Fig. 4. Effect of the MeOH content on (a) retention factor and enantioselectivity; (b) enantioseparation and column efficiency; effect of the (c) concentration and (d) ratio of the HAc/TEA for B-leucine retention factor and enantioresolution in the polar organic phase mode. Experimental conditions: (a) and (b) MeOH/ACN/HAc/TEA (at the desired ratio of MeOH and ACN/0.2/0.04, v/v/v/v); (c) MeOH/ACN/HAc/TEA (40/60/at the desired content of HAc and TEA, v/v/v/v); (d) MeOH/ACN/HAc/TEA (40/60/at the desired ratio of HAc and TEA, v/v/v/v). Column dimensions: 15 cm × 100 μm I.D.; UV detection wavelength: 254 nm; flow rate: 10 μL/min; injection volume: 20 nL.

Under the optimal POM conditions, this is, MeOH/ACN (40/60, v/v), 0.055% (v/v) HAc and 0.005% (v/v) TEA (i.e., a 11:1 HAc/TEA (%, v/v) ratio), 52 N-derivatized amino acids were tested. It is important to note that the peaks of 3,5-DCIB-methionine, 3,5-DMB-threonine, *m*-CIB-threonine and B-threonine were deteriorated when using the optimal POM conditions (see **Fig. S4**), thus, mobile phase conditions had to be slightly modified. As can be seen in **Table 3**, also 44 out of 52 were finally baseline enantioseparated (*Rs* > 1.5) on the monolithic column using POM conditions. Same as in RPM, most of FMOC-derivatized amino acids also can not be baseline enantioresolved. The enantioseparations for *p*-NB-leucine, *p*-CIB-alanine, *p*-CIB-methionine, 3,5-DMB-leucine, 3,5-DCIB-alanine and *p*-NB-methionine under POM conditions were displayed in **Fig. 5**.

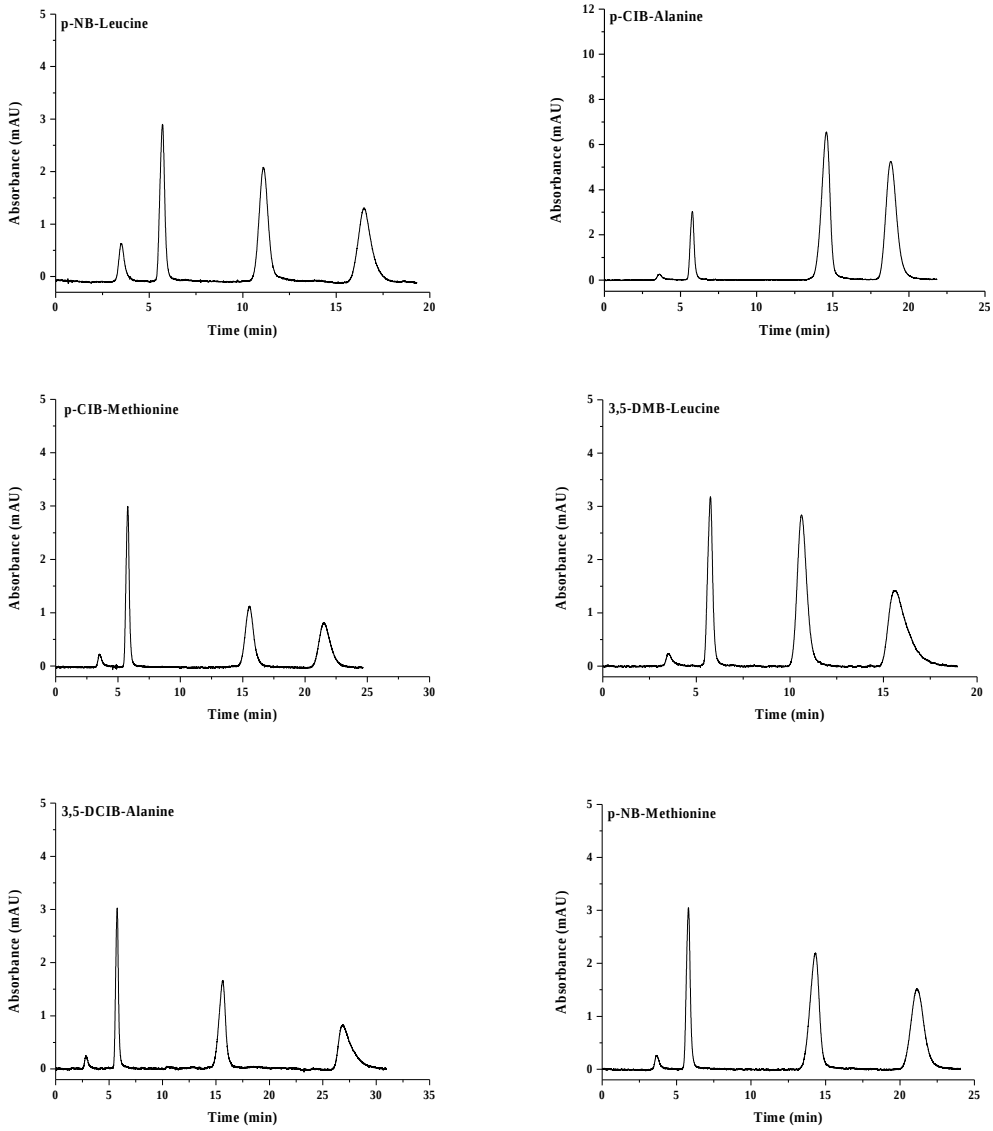


Fig. 5. Enantioseparation of some N-derivatized amino acids in the polar organic phase mode. Mobile phase: ACN/MeOH/HAc/TEA (60/40/0.055/0.005, v/v/v/v); Other experimental conditions are the same as in **Fig. 4**.

Table 3. Enantioseparation of *N*-derivatized amino acids in the polar organic phase mode.

Sample	k_1	k_2	α	*Rs*	N_1 (m)	N_2 (m)
3,5-DNB-isoleucine[a]	1.07	7.97	7.45	14.30	21100	18500
3,5-DNB-leucine[a]	1.03	5.00	4.85	11.03	21700	16900
3,5-DNB-valine[a]	1.19	6.27	5.27	9.79	22000	12300
3,5-DNB-norvaline[a]	1.16	4.87	4.20	9.35	26000	13500
3,5-DNB-norleucine[a]	1.15	4.94	4.29	8.98	16600	10000

3,5-DNB-methionine[a]	1.61	6.41	3.98	8.86	18800	13400
3,5-DNB-tryptophan[a]	1.94	7.66	3.95	7.87	12500	8950
3,5-DNB-citrulline[a]	1.28	4.79	3.74	7.84	15500	12300
3,5-DNB-methionine sulfone[a]	1.66	5.67	3.42	7.72	11700	11400
3,5-DNB-phenylalanine[a]	1.66	6.32	3.81	5.98	11600	8000
3,5-DNB-alanine[a]	1.25	2.15	1.72	3.90	30700	28300
3,5-DCIB-threonine[a]	2.28	5.13	2.25	6.87	28100	24700
3,5-DCIB-norvaline[a]	1.40	3.89	2.78	6.48	17200	16200
3,5-DCIB-valine[a]	1.65	4.59	2.78	6.47	18600	14100
3,5-DCIB-serine[a]	2.52	5.24	2.08	6.19	23400	21600
3,5-DCIB-alanine[a]	1.71	3.67	2.15	5.89	29300	26400
3,5-DCIB-isoleucine[a]	1.47	4.67	3.18	5.72	21300	9600
3,5-DCIB-norleucine[a]	1.37	3.46	2.53	5.22	18700	14900
3,5-DCIB-methionine[b]	1.52	3.64	2.39	5.34	21500	14400
3,5-DCIB-phenylalanine[a]	2.28	5.12	2.25	5.15	19500	15600
3,5-DCIB-leucine[a]	1.19	3.16	2.66	5.13	22100	13100
3,5-DCIB-cysteine[a]	4.38	7.67	1.75	4.99	21200	19000
3,5-DCIB-tryptophan[a]	2.32	4.91	2.12	4.56	16200	9200
3,5-DCIB-methionine sulfone[a]	1.95	4.50	2.31	4.56	18900	7000
3,5-DCIB-citrulline[a]	1.56	4.18	2.68	3.67	7800	2400
p-NB-leucine[a]	0.94	1.89	2.01	4.18	24300	21600
p-NB-methionine[a]	1.47	2.65	1.80	4.13	23800	21700
p-NB-alanine[a]	1.35	2.21	1.64	3.64	29300	27700
3,5-DMB-alanine[a]	1.24	2.12	1.71	3.84	31400	29200
3,5-DMB-methionine[a]	1.54	2.73	1.77	3.68	24200	18900

3,5-DMB-threonine[b]	1.24	2.18	1.76	3.67	25000	18900
3,5-DMB-leucine[a]	0.84	1.71	2.04	3.14	20400	12400
3,5-DMB-norvaline[a]	1.00	1.91	1.91	2.75	14900	10900
3,5-DMB-norleucine[a]	1.03	1.82	1.77	2.61	17900	13500
m-CIB-methionine[a]	1.78	3.06	1.72	3.96	21600	17700
m-CIB-alanine[a]	1.59	2.52	1.58	3.74	31100	28900
m-CIB-leucine[a]	1.08	1.98	1.83	3.61	23300	21800
m-CIB-threonine[c]	1.18	2.09	1.77	3.33	15700	12300
p-CIB-leucine[a]	1.01	1.74	1.72	3.63	23100	16800
p-CIB-methionine[a]	1.69	2.72	1.61	3.45	25500	23900
p-CIB-alanine[a]	1.52	2.26	1.49	3.06	31800	28300
B-methionine[a]	1.65	2.26	1.37	2.23	22000	19300
B-threonine[c]	1.06	1.48	1.40	2.08	32100	28800
B-leucine[a]	0.93	1.28	1.38	1.61	22500	17000
FMOC-alanine[a]	1.15	1.36	1.18	0.78	16200	15900
FMOC-tryptophan[a]	1.71	2.04	1.19	0.69	11100	6700
FMOC-methionine[a]	0.96	1.23	1.28	0.96	15000	8500
FMOC-valine[a]	0.70	0.97	1.39	1.26	19400	4000
FMOC-norvaline[a]	0.68	0.86	1.26	0.94	21600	16400
FMOC-norleucine[a]	0.69	0.87	1.26	0.96	22000	17500
FMOC-methionine sulfone[a]	1.13	1.42	1.25	1.01	25000	9500
FMOC-isoleucine[a]	0.66	0.99	1.50	/	/	/

Experimental conditions: (a) ACN/MeOH/HAc/TEA (60/40/0.055/0.005, v/v/v/v); (b) ACN/MeOH/HAc/TEA (60/40/0.11/0.01, v/v/v/v); (c) ACN/MeOH/HAc/TEA (60/40/0.22/0.02, v/v/v/v). Other experimental conditions as in **Fig. 5**.

3.3. Comparison of the enantioseparation of N-derivatized amino acids under POM and RPM conditions

In order to further investigate the enantioseparation ability of the MQD-silica hybrid monolithic column for the 52 *N*-derivatized amino acids, the results obtained under RPM and POM conditions were compared in terms of the α, *Rs*, N_1 and analysis time.

A total of 44 N-derivatized amino acids could be enantioseparated in both RPM and POM with similar α values (**Fig. S5**). Comparing the α values for all the amino acids in the same mode, the higher electrophilic character of the N-protecting groups would lead to a higher enantioselectivity, following this trend: 3,5-DNB-leucine (α = 4.22)> 3,5-DCIB-leucine (α = 2.62)> 3,5-DMB-leucine (α = 1.91)> *m*-CIB-leucine (α = 1.80)> *p*-NB-leucine (α = 1.73)> *p*-CIB-leucine (α=1.58)> B-leucine (α=1.33) (in the case of RPM; similar trend was found for POM). Regarding the enantioresolution and column efficiency (**Fig. S6**), these values were higher in POM than in RPM. Unfortunately, most of FMOC-derivatized amino acids were not baseline enantioseparated in any of the two modes (except the FMOC-isoleucine and FMOC-valine which could be baseline enantioseparated in RPM), as earlier commented. Regarding the analysis time values, they were shorter in POM when compared to RPM for all the N-derivatized amino acids tested (**Fig. S5**).

Overall, the POM offered better performance than RPM for the N-derivatized amino acids herein tested in terms of the enantioresolution, efficiency and analysis time. Under the POM conditions, higher column efficiency was observed on the MQD-silica hybrid monolithic column than the poly (MQD-*co*-HEMA-*co*-EDMA) monolithic column [4] for almost all the tested compounds, for example the theoretical plate number for 3,5-DNB-alanine are 30700 plates/m and 28300 plates/m on the MQD-silica hybrid monolith, while they are 10700 and 7400 plates/m on the poly (MQD-*co*-HEMA-*co*-EDMA) monolith (see **Fig. S7**).

4. Conclusion

This study shows a straightforward “one-step” strategy to prepare a MQD-silica hybrid monolithic column within a 100 μm I.D. capillary. Under the optimal preparation conditions, the resulting monolithic column exhibited good permeability, mechanical stability, and reproducibility. After the optimization of the both RPM and POM chromatographic conditions, a total of 52 N-derivatized amino acids were enantioseparated in nano-LC, and baseline enantioseparation was observed for 44 of

them in both modes. Most of FMOC-derivatized amino acids were not baseline separated under both chromatographic modes although chiral discrimination was observed for all of them. When comparing RPM and POM chromatographic modes, the POM offered better performance than RPM in terms of column efficiency and enantioresolution with shorter analysis time.

Appendix C.

Supplemental material for article 3.

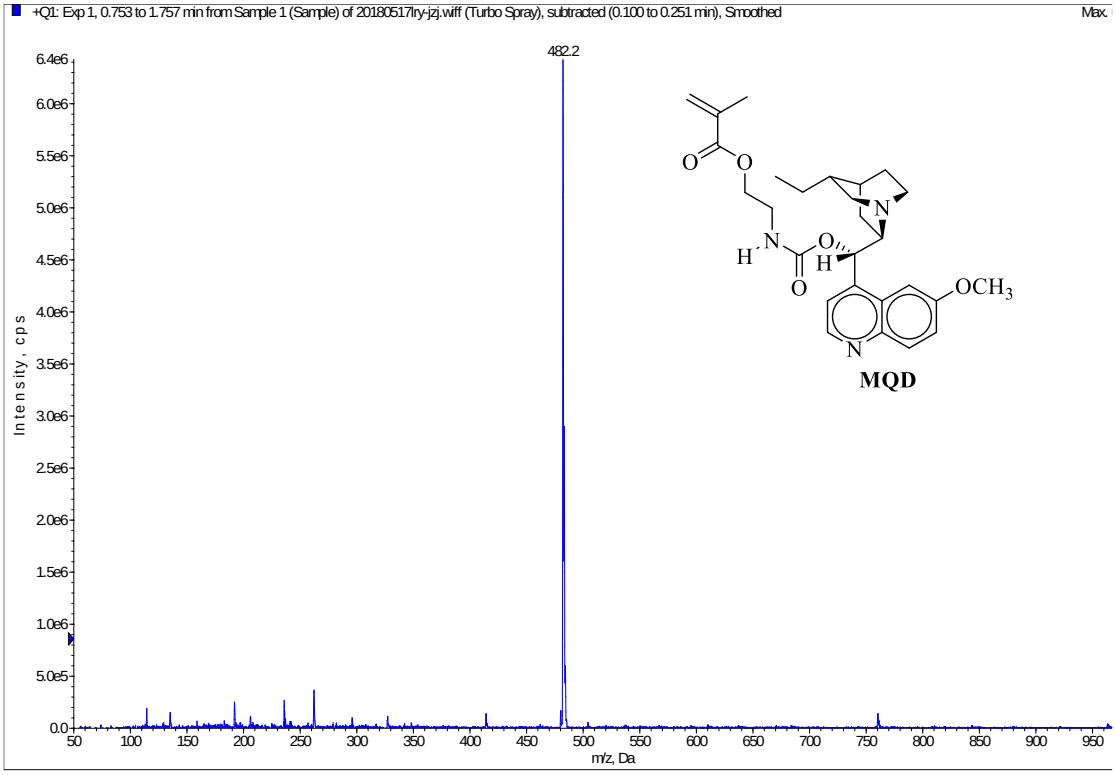


Fig. S1. ESI-MS spectrum of the MQD monomer.

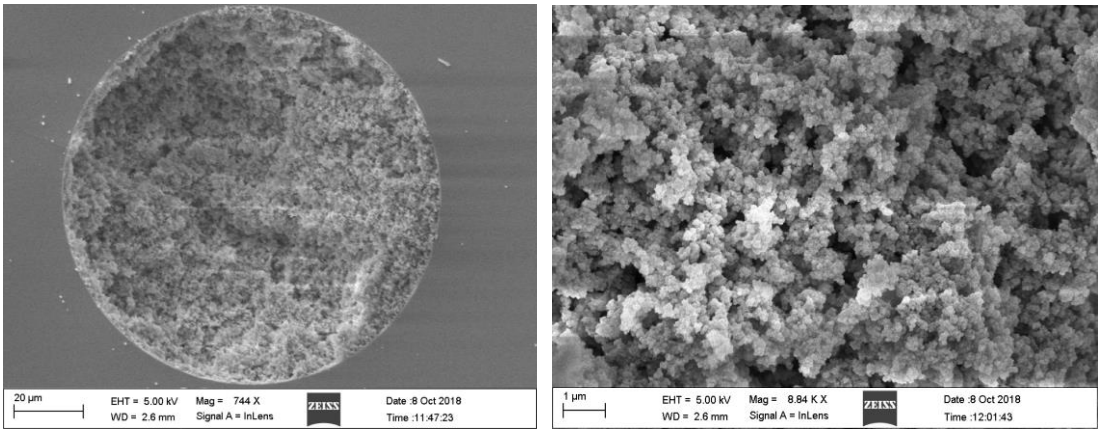


Fig. S2. SEM images of the MQD-silica monolithic column at different magnifications.

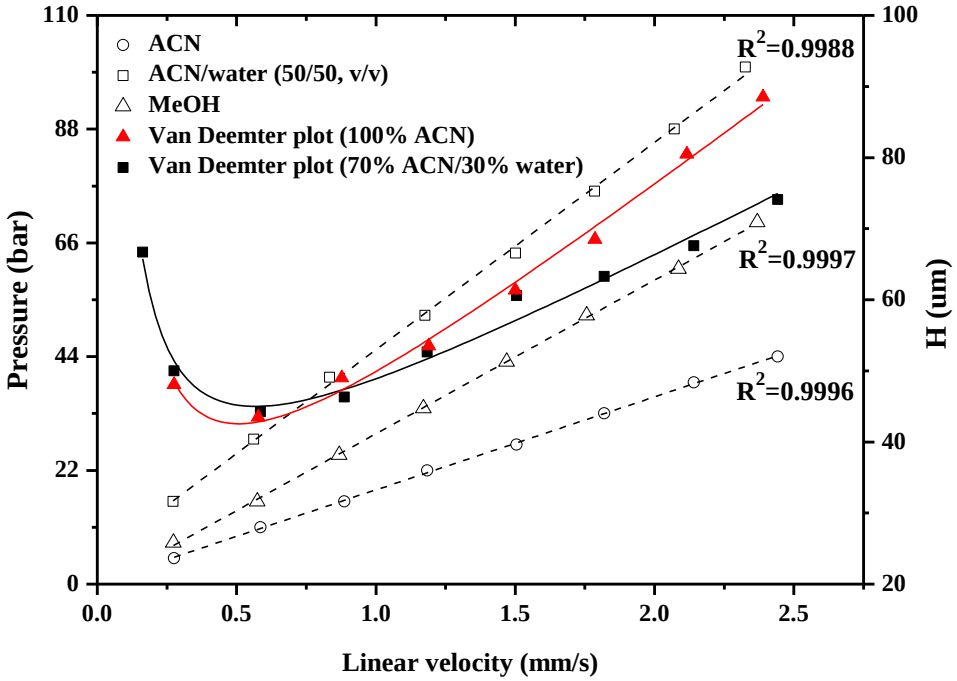
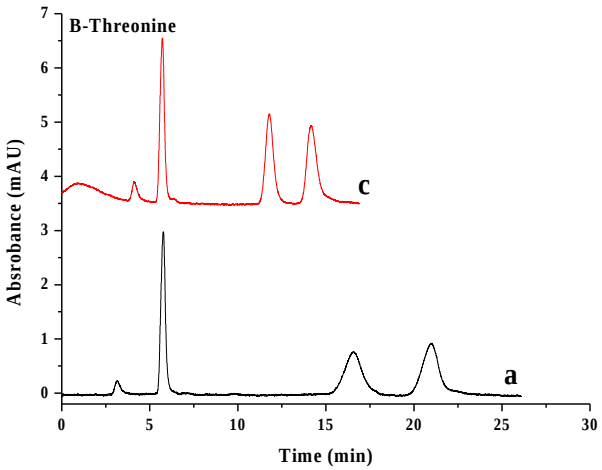


Fig. S3. Dependence of backpressure on linear velocity and Van Deemter plots for the MQD-silica hybrid monolithic column. Experimental conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase for Van Deemter plots test: 100% ACN (POM), and 30% H_2O/70% ACN (RPM), UV detection wavelength: 214 nm; injection volume: 20 nL; samples: Toluene (100% ACN or 100% MeOH as mobile phase) and thiourea (H_2O/ACN (50/50, v/v) or H_2O/ACN (30/70, v/v) as mobile phase)



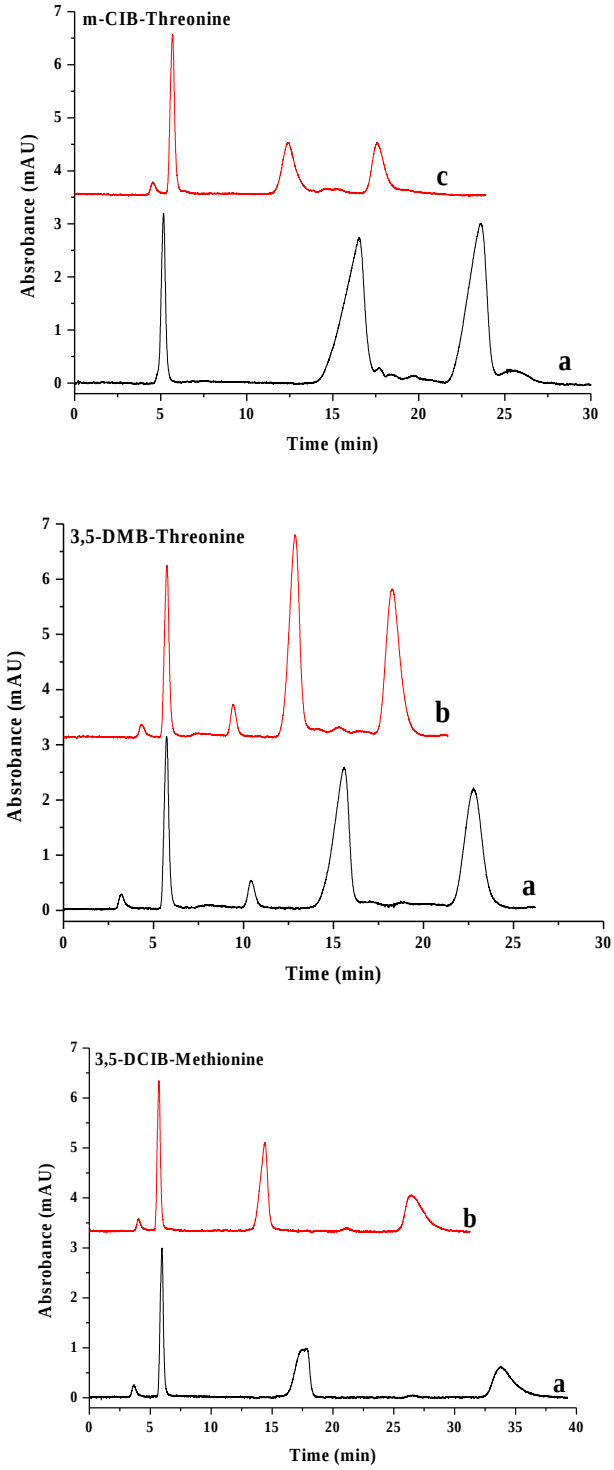


Fig. S4. Comparison of the different conditions in POM for B-threonine, *m*-CIB-threonine, 3,5-DMB-threonine and 3,5-DCIB-methionine. Experimental conditions: (a) ACN/MeOH/HAc/TEA (60/40/0.055/0.005, v/v/v/v); (b) ACN/MeOH/HAc/TEA (60/40/0.11/0.01, v/v/v/v); (c) ACN/MeOH/HAc/TEA (60/40/0.22/0.02, v/v/v/v). Column dimensions: 15 cm × 100 μm I.D.; UV detection wavelength: 254 nm; flow rate: 10 μL/min; injection volume: 20 nL.

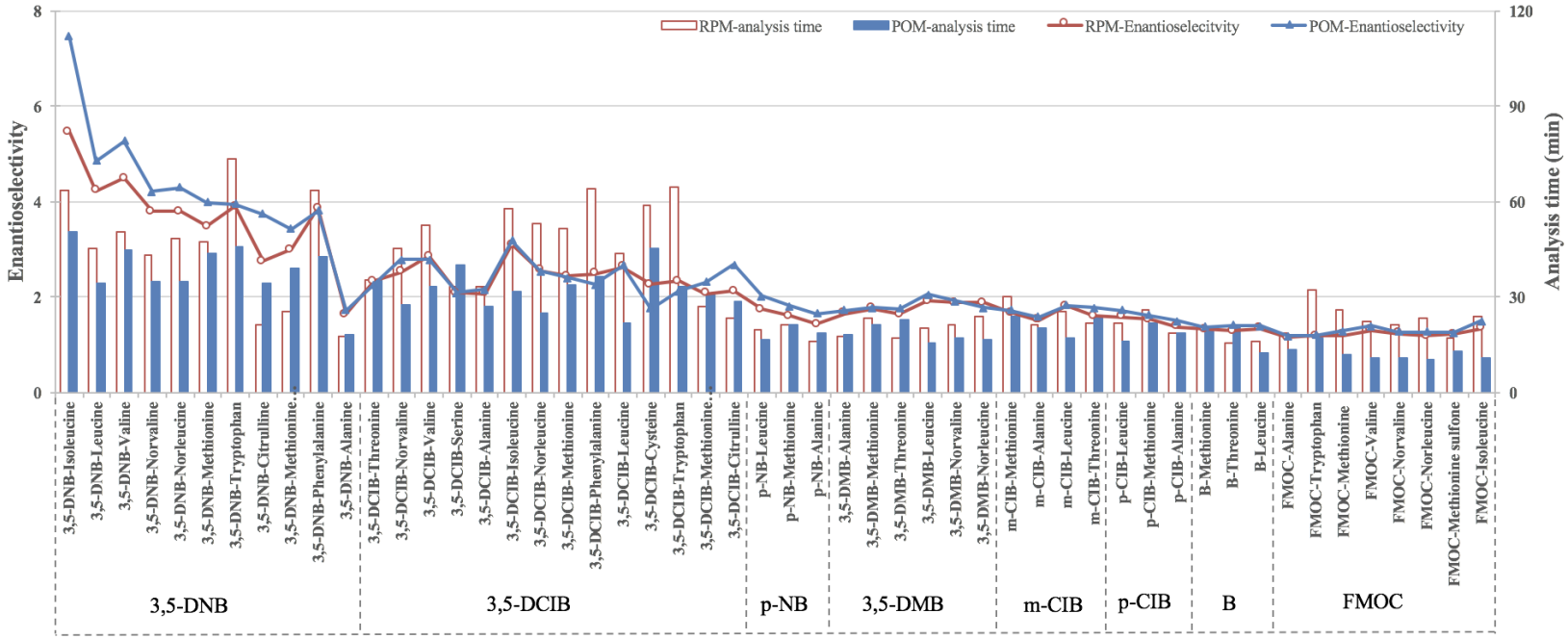


Fig. S5. Comparison of the enantioselectivity and analysis time (time of the second enantiomer) under the reversed phase mode and polar organic phase mode. Experimental conditions for reversed phase mode: ACN/10 mM ammonium acetate (70/30, v/v) (apparent pH=5.3); and polar organic mode: (a) ACN/MeOH/HAc/TEA (60/40/0.055/0.005, v/v/v/v); (b) ACN/MeOH/HAc/TEA (60/40/0.11/0.01, v/v/v/v); (c) ACN/MeOH/HAc/TEA (60/40/0.22/0.02, v/v/v/v). Other experimental conditions as in **Fig. S4**.

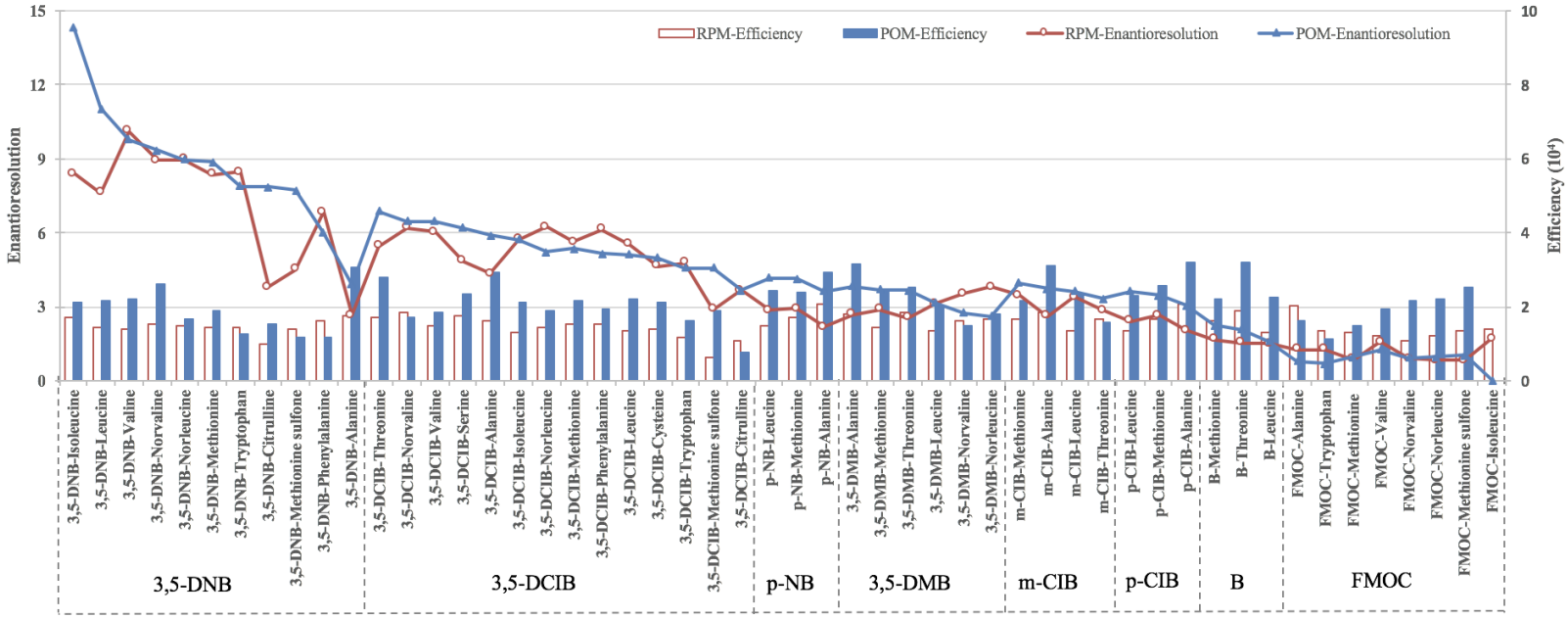


Fig. S6. Comparison of the enantioresolution and efficiency under the reversed phase mode and the polar organic phase mode. Experimental conditions for reversed phase mode and polar organic mode as in **Figure S5**.

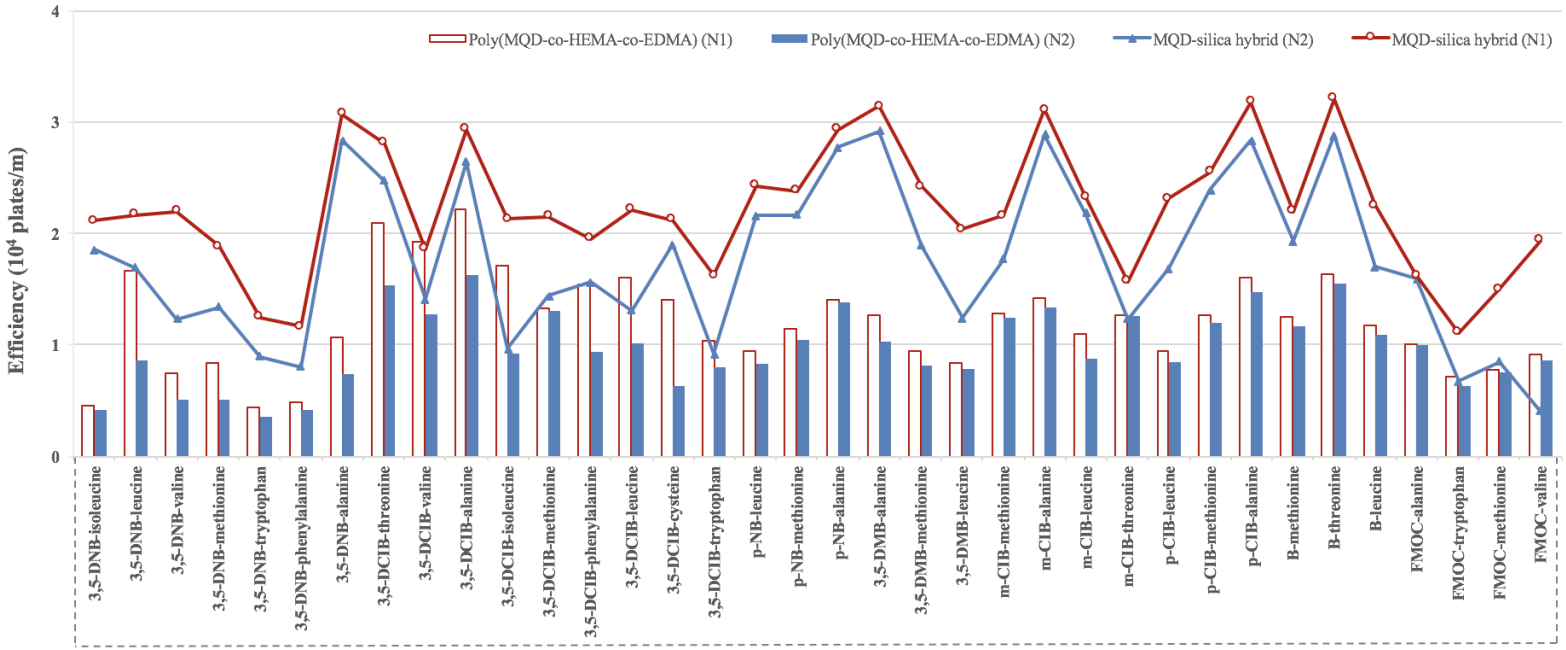


Fig. S7. Comparison of the efficiency with the poly (MQD-*co*-HEMA-*co*-EDMA) under the polar organic phase mode. Experimental conditions for MQD-silica hybrid monolith as in **Fig. S5**. The conditions for poly (MQD-*co*-HEMA-*co*-EDMA) as in Ref. [4].

Table S1. Permeability of the MQD-silica hybrid monolithic column

Mobile phase	Relative polarity	Viscosity η ($\times 10^{-3}$ Pa·s)	Permeability K ($\times 10^{-14}$ m^2)
ACN	0.460	0.369	2.95
MeOH	0.762	0.544	2.93
ACN/H_2O (50:50, v/v)	/	0.820	2.47

Relative polarity and viscosity data of pure liquids were obtained from Ref. [23,24].

Table S2. Reproducibility of the MQD-silica hybrid monolithic column

	Average retention factor (RSD)		Average selectivity α (RSD)	Average resolution *Rs* (RSD)
	k_1	k_2		
Run to run (n=6)	1.51 (1.81%)	2.63 (1.24%)	1.74 (1.64%)	2.83 (2.07%)
Column to column (n=5)	1.54 (2.53%)	2.64 (3.05%)	1.72 (3.04%)	2.85 (3.90%)
Batch to batch (n=3)	1.52 (4.29%)	2.66 (3.59%)	1.76 (4.04%)	2.84 (2.88%)
Day to day (n=3)	1.53 (1.73%)	2.67 (1.69%)	1.75 (1.10%)	2.85 (1.07%)

Experimental conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: 10 mM ammonium acetate/ACN (30/70, v/v) (apparent pH=5.3); UV detection wavelength: 254 nm; injection volume: 20 nL; sample: *p*-NB-leucine.

Table S3. Effect of mobile phase composition on enantioseparation for 3,5-DMB-leucine under the reversed phase mode

Condition	Sample	k_1	k_2	α	R_s	N_1 (m)	N_2 (m)
Effect of the apparent pH[a]							
pH=4.3	3,5-DMB-leucine	0.29	0.54	1.86	1.51	14700	12200
pH=4.8	3,5-DMB-leucine	0.59	1.15	1.95	2.12	13000	9870
pH=5.3	3,5-DMB-leucine	1.19	2.32	1.95	2.71	12050	8570
pH=5.8	3,5-DMB-leucine	1.79	3.40	1.90	2.77	9300	8200
pH=6.3	3,5-DMB-leucine	1.88	3.31	1.76	2.66	9050	8700
Effect of ACN content[b]							
60%	3,5-DMB-leucine	3.76	7.30	1.94	3.98	12400	10800
70%	3,5-DMB-leucine	2.36	4.59	1.94	3.40	12000	9000
80%	3,5-DMB-leucine	1.19	2.32	1.95	2.71	12050	8570
90%	3,5-DMB-leucine	0.70	1.32	1.89	2.58	14000	12700
Effect of the buffer concentration[c]							
1 mM	3,5-DMB-leucine	5.14	9.97	1.94	4.02	12000	9900
2.5 mM	3,5-DMB-leucine	3.05	5.95	1.95	3.92	12300	11300
5.0 mM	3,5-DMB-leucine	2.36	4.59	1.94	3.40	12000	9000
10.0 mM	3,5-DMB-leucine	1.37	2.62	1.91	3.11	13500	10900
15.0 mM	3,5-DMB-leucine	0.97	1.98	2.04	2.87	12400	10200
30.0 mM	3,5-DMB-leucine	0.63	1.25	1.98	2.39	13900	11400

Experimental conditions: (a) 5 mM ammonium acetate/ACN (20/80, v/v) (at the desired apparent pH values); (b) 5 mM ammonium acetate/ACN (at the desired ratio of ACN and buffer, v/v) (apparent pH=5.3); (c) ammonium acetate/ACN (at the desired concentration of the buffer) (30/70, v/v) (apparent pH=5.3). Column dimensions: 15 cm × 100 μm I.D.; UV detection wavelength: 254 nm; flow rate: 10 μL/min; injection volume: 20 nL.

Table S4. Effect of mobile phase composition on enantioseparation for 3,5-DCIB-leucine under the polar organic phase mode.

Condition	Sample	k_1	k_2	α	R_s	N_1 (m)	N_2 (m)
Effect of the volume ratio of MeOH/ACN[a]							
60:40 (v/v)	3,5-DCIB-leucine	0.45	1.33	2.96	4.09	26500	13600
50:50 (v/v)	3,5-DCIB-leucine	0.49	1.46	2.98	4.30	25800	14000
40:60 (v/v)	3,5-DCIB-leucine	0.50	1.47	2.94	4.29	24100	14200
30:70 (v/v)	3,5-DCIB-leucine	0.58	1.56	2.69	3.91	25400	11500
20:80 (v/v)	3,5-DCIB-leucine	0.72	1.71	2.38	3.85	30100	12700
Effect of the HAc+TEA total content (v/v)[b]							
0.015 % (0.0125% HAc +0.0025% TEA)	3,5-DCIB-leucine	1.36	3.23	2.38	4.38	8200	18400
0.03% (0.025% HAc + 0.005% TEA)	3,5-DCIB-leucine	1.02	2.57	2.52	4.42	13900	14100
0.06% (0.05% HAc + 0.01% TEA)	3,5-DCIB-leucine	0.87	2.26	2.60	4.51	20000	13200
0.12% (0.1% HAc + 0.02% TEA)	3,5-DCIB-leucine	0.67	1.79	2.67	4.37	23000	13800
0.24% (0.2% HAc + 0.04% TEA)	3,5-DCIB-leucine	0.50	1.47	2.94	4.29	24100	14200
0.48% (0.4% HAc + 0.08% TEA)	3,5-DCIB-leucine	0.36	1.03	2.86	3.92	34100	17100
Effect of the ratio of HAc/TEA[c]							
3:1 (v/v)	3,5-DCIB-leucine	0.67	1.69	2.52	4.24	18600	17800
5:1 (v/v)	3,5-DCIB-leucine	0.87	2.26	2.60	4.51	20000	13200
11:1 (v/v)	3,5-DCIB-leucine	1.19	3.16	2.66	5.13	22100	13100
29:1 (v/v)	3,5-DCIB-leucine	1.43	3.86	2.70	5.12	22900	11000

Experimental conditions: (a) MeOH/ACN/HAc/TEA (at the desired ratio of MeOH and ACN/0.2/0.04, v/v/v/v); (b) MeOH/ACN/HAc/TEA (40/60/at the desired content of HAc and TEA, v/v/v/v); (c) MeOH/ACN/HAc/TEA (40/60/at the desired ratio of HAc and TEA, v/v/v/v). Column dimensions: 15 cm × 100 μm I.D.; UV detection wavelength: 254 nm; flow rate: 10 μL/min; injection volume: 20 nL.

Acknowledgements

M. L. Marina and E. Sánchez-López thank the Ministry of Economy and Competitiveness (Spain) for research projects CTQ2013-48740-P and CTQ2016-76368-P. D. Xu and E. Sánchez-López thank the Comunidad of Madrid and European funding from FEDER program for their contracts (AVANSECAL-CM program, ref. S2013/ABI-3028).

References

[1] M. Lämmerhofer, E.C. Peters, C. Yu, F. Svec, J.M.J. Fréchet, Chiral monolithic columns for enantioselective capillary electrochromatography prepared by copolymerization of a monomer with quinidine functionality. 1. Optimization of polymerization conditions, porous properties, and chemistry of the stationary phase, Anal. Chem. 72 (2000) 4614-4622.

[2] M. Lämmerhofer, E. Tobler, E. Zarbl, W. Lindner, F. Svec, J.M.J. Fréchet, Macroporous monolithic chiral stationary phases for capillary electrochromatography: New chiral monomer derived from cinchona alkaloid with enhanced enantioselectivity, Electrophoresis 24 (2003) 2986-2999.

[3] Q.Q. Wang, P.J. Zhu, M. Ruan, H.H. Wu, K. Peng, H. Han, G. W. Somsen, J. Crommen, Z.J. Jiang, Chiral separation of acidic compounds using an *O*-9-(*tert*-butylcarbamoyl) quinidine functionalized monolith in micro-liquid chromatography, J. Chromatogr. A 1444 (2016) 64-73.

[4] Q.Q. Wang, J. Feng, H. Han, P.J. Zhu, H.H. Wu, M.L. Marina, J. Crommen, Z.J. Jiang, Enantioseparation of N-derivatized amino acids by micro-liquid chromatography using carbamoylated quinidine functionalized monolithic stationary phase, J. Chromatogr. A 1363 (2014) 207-215.

[5] H.H. Wu, Q.Q. Wang, M. Ruan, K. Peng, P.J. Zhu, J. Crommen, H. Hai, Z.J. Jiang, Enantioseparation of N-derivatized amino acids by micro-liquid chromatography/laser induced fluorescence detection using quinidine-based monolithic columns, J. Pharm. Biomed. Anal. 121 (2016) 244-252.

[6] Q.Q. Wang, E. Sánchez-López, H. Han, H.H. Wu, P.J. Zhu, J. Crommen, M.L. Marina, Z.J. Jiang, Separation of N-derivatized di- and tri-peptide stereoisomers by micro-liquid chromatography using a quinidine-based monolithic column - Analysis of l-carnosine in dietary supplements, J. Chromatogr. A 1428 (2016) 176-184.

[7] L.N. Tran, J.H. Park, Enantiomer separation of acidic chiral compounds on a quinine-silica/zirconia hybrid monolith by capillary electrochromatography, J. Chromatogr. A 1396 (2015) 140-147.
[8] L.N. Tran, J.A. Jeong, J.H. Park, Enantiomer separation of acidic chiral compounds on a *tert*-butylcarbamoylquinine-silica hybrid monolith by capillary electrochromatography, Bull. Korean Chem. Soc. 37 (2016) 1050-1056.
[9] M. Lämmerhofer, F. Svec, J.M.J. Fréchet, Chiral monolithic columns for enantioselective capillary electrochromatography prepared by copolymerization of a monomer with quinidine functionality. 2. Effect of chromatographic conditions on the chiral separations, Anal. Chem. 72 (2000) 4623-4628.
[10] M. Lämmerhofer, F. Svec, J.M.J. Fréchet, W. Lindner, Monolithic stationary phases for enantioselective capillary electrochromatography, J. Micro. Sep. 12 (2000) 597-602.
[11] B. Preinerstorfer, W. Bicker, W. Lindner, M. Lämmerhofer, Development of reactive thiol-modified monolithic capillaries and in-column surface functionalization by radical addition of a chromatographic ligand for capillary electrochromatography, J. Chromatogr. A 1044 (2004) 187-199.
[12] E.J. Carrasco-Correa, G. Ramis-Ramos, J.M. Herrero-Martínez, M. Lämmerhofer, Polymethacrylate monoliths with immobilized poly-3-mercaptopropyl methylsiloxane film for high-coverage surface functionalization by thiol-ene click reaction, J. Chromatogr. A 1367 (2014) 123-130.
[13] M. Wolter, M. Lämmerhofer, In-situ functionalized monolithic polysiloxane polymethacrylate composite materials from polythiol-ene double click reaction in capillary column format for enantioselective nano-high-performance liquid chromatography, J. Chromatogr. A 1497 (2017) 172-179.
[14] S. Buchinger, B. Follrich, M. Lämmerhofer, D. Lubda, W. Lindner, Chirally functionalized anion-exchange type silica monolith for enantiomer separation of 2-aryloxypropionic acid herbicides by non- aqueous capillary electrochromatography, Electrophoresis 30 (2009) 3804-3813.
[15] B. Preinerstorfer, D. Lubda, A. Mucha, P. Kafarski, W. Lindner, M. Lämmerhofer, Stereoselective separations of chiral phosphinic acid pseudodipeptides by CEC using silica monoliths modified with an anion-exchange-type chiral selector, Electrophoresis 27 (2006) 4312-4320.
[16] Q.X. Zhang, V. Gil, E. Sánchez-López, M.A. García, Z.J. Jiang, M.L. Marina,

Evaluation of the potential of a quinidine-based monolithic column on the enantiomeric separation of herbicides by nano-liquid chromatography, Microchem. J. 123 (2015) 15-21.

[17] H. Lin, J. Ou, Z. Zhang, J. Dong, M. Wu, H. Zou, Facile preparation of zwitterionic organic-silica hybrid monolithic capillary column with an improved "one-pot" approach for hydrophilic-interaction liquid chromatography (HILIC), Anal. Chem. 84 (2012) 2721-2728.

[18] Z. Zhang, M. Wu, R. Wu, J. Dong, J. Ou, H. Zou, Preparation of perphenylcarbamoylated β-cyclodextrin-silica hybrid monolithic column with "one-pot" approach for enantioseparation by capillary liquid chromatography, Anal. Chem. 83 (2011) 3616-3622.

[19] H. Zhang, J. Ou, Z. Lui, H. Wang, Y. Wei, H. Zou, Preparation of hybrid monolithic columns via "one-pot" photoinitiated thiol-acrylate polymerization for retention-independent performance in capillary liquid chromatography, Anal. Chem. 87 (2015) 8789-8797.

[20] J. Ou, H. Lin, Z. Zhang, G. Huang, J. Dong, H. Zou, Recent advances in preparation and application of hybrid organic-silica monolithic capillary columns, Electrophoresis 34 (2013) 126-140.

[21] D.S. Domingues, I.D. de Souza, M.E.C. Queiroz, Analysis of drugs in plasma samples from schizophrenic patients by column-switching liquid chromatography-tandem mass spectrometry with organic-inorganic hybrid cyanopropyl monolithic column, J. Chromatogr. B 993-994 (2015) 26-35.

[22] H. Zhao, H. Lyu, W. Qin, Z. Xie, Synthesis of boronate-functionalized organic-inorganic hybrid monolithic column for the separation of cis-diol containing compounds at low pH, Electrophoresis 39 (2018) 924-932.

[23] Z. Zhang, H. Lin, J. Ou, H. Qin, R, Wu, J. Dong, H, Zou, Preparation of phenyl-silica hybrid monolithic column with "one-pot" process for capillary liquid chromatography, J. Chromatogr. A 1228 (2012) 263-269.

[24] Z.J. Jiang, N.W. Smith, P.D. Ferguson, M.R. Taylor, Hydrophilic interaction chromatography using methacrylate-based monolithic capillary column for the separation of polar analytes, Anal. Chem. 79 (2007) 1243-1250.

[25] J. Lin, S. Liu, J. Lin, X. Lin, Z. Xie, Novel highly hydrophilic methacrylate-based monolithic column with mixed-mode of hydrophilic and strong cation-exchange interactions for pressurized capillary electrochromatography, J. Chromatogr. A 1218

(2011) 4671-4677.
[26] D. Xu, H. Shao, R. Luo, Q. Wang, E. Sánchez-López, S. Fanali, M.L. Marina, Z. Jiang, A facile and efficient single-step approach for the fabrication of vancomycin functionalized polymer-based monolith as chiral stationary phase for nano-liquid chromatography, J. Chromatogr. A 1557 (2018) 43-50.
[27] M.L. Hsieh, L.K. Chau, Y.S. Hon, Single-step approach for fabrication of vancomycin-bonded silica monolith as chiral stationary phase, J. Chromatogr. A 1358 (2014) 208-216.
[28] X.L. Dong, J. Dong, J.J. Ou, Y. Zhu, H.F. Zou, Preparation and evaluation of a vancomycin immobilized silica monolith as chiral stationary phase for CEC, Electrophoresis 28 (2007) 2606-2612.

III. 4.

Enantioseparation of FMOC-amino acids using an O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine-silica hybrid monolithic column by nano-LC. Quantitation of L-norvaline and L-tryptophan in dietary supplements

III.4. Enantioseparation of FMOC-amino acids using an O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine-silica hybrid monolithic column by nano-LC. Quantitation of L-norvaline and L-tryptophan in dietary supplements

III.4.1. Preface

Amino acids are essential and ubiquitous compounds playing a vital role in many living organisms. Differences in the properties of D- and L-enantiomers have been widely reported, both for protein and non-protein amino acids. For instance, the L-enantiomer of the non-protein amino acid norvaline enhances the nitric oxide production, which is an important regulator and mediator in physiological and pathophysiological events such as vasodilatation. However, to the best of our knowledge, D-norvaline has not been reported as an effective substance. On the other hand, L-tryptophan is the precursor of important neurotransmitters, hormones and other relevant biomolecules. The level of L-tryptophan in humans has been reported to act as a biomarker of certain diseases. At the pharmacological level, L-tryptophan is used as antidepressant agent, whereas D-tryptophan is considered as an impurity because of its low biological activity. Due to their interesting properties, amino acids can be used as ingredients in dietary supplements where the presence of the D-enantiomer is not allowed by legal regulations. Therefore, the enantiomeric determination of DL-amino acids in real samples remains as a very important challenge.

In order to obtain satisfactory chiral separation of amino acids, various chiral stationary phases (CSPs) have been developed. Cinchona alkaloids, especially quinine and quinidine have attracted much attention owing to their excellent enantioselectivity for amino acids. Usually, because of the low detection sensitivity and weak interaction between the chiral selector and analytes, different derivatization groups were used to react with amino acids. Among the different derivatization reagents, FMOC has the advantage of reacting with primary and secondary amines in just 3 min.

In section III.3 of this PhD Thesis, the preparation of a MQD-silica hybrid monolithic column has been described and the enantiomeric separation of protein and non-protein amino acids has been investigated using up to 8 different derivatization reagents. Nevertheless, from the eight FMOC-amino acids tested, only two of them were baseline separated in the reversed phase mode and none of them was baseline separated in the polar organic phase mode. Moreover, the applicability of quinidine silica-hybrid monolithic columns to the quantitative analysis of amino acids in real samples has not

been demonstrated yet. For this reason, in this chapter, the quinidine silica hybrid monolithic column prepared in this PhD Thesis was applied to the development of an analytical methodology enabling the enantiomeric separation of FMOC-amino acids and the enantiomeric determination of L-norvaline and L-tryptophan in dietary supplements.

III.4.2. Objectives

The specific objectives of this work were:

- To investigate the enantioseparation ability of the quinidine silica-hybrid monolithic column developed for FMOC-amino acids in both reversed-phase and polar organic-phase modes.
- To systematically optimize the chromatographic conditions to enantioseparate the N-derivatized amino acids.
- To develop an analytical methodology enabling the enantiomeric separation of FMOC-amino acids and the determination of L-norvaline and L-tryptophan in dietary supplements.
- To evaluate the analytical characteristics of the developed method in terms of linearity, precision, accuracy, LODs and LOQs.
- To quantitate L-tryptophan and L-norvaline in different dietary supplements.

III.4.3. Results

The results obtained in this work are included in the following scientific article:

Article 4: *Enantioseparation of amino acids using an O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine-silica hybrid monolithic column by nano-LC. Quantitation of L-norvaline and L-tryptophan in dietary supplements*

D. Xu, E. Sánchez-López, Q. Wang, Z. Jiang, M. L. Marina

J. Pharm. Anal., DOI: 10.1016/j.pha.2019.10.001.

Article 4

Enantioseparation of FMOC-amino acids using an O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine-silica hybrid monolithic column by nano-LC. Quantitation of L-norvaline and L-tryptophan in dietary supplements

D. Xu, E. Sánchez-López, Q. Wang, Z. Jiang, M. L. Marina

J. Pharm. Anal., DOI:10.1016/j.pha.2019.10.001.

Abstract

An analytical methodology based on an O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine (MQD)-silica hybrid monolithic column was developed for the enantioseparation of 9-fluorenylmethoxycarbonyl (FMOC) derivatized amino acids by nano-liquid chromatography. The mobile phase was optimized including the apparent pH, content of ACN, and concentration of the buffer to obtain a satisfactory enantioresolution performance. 27 FMOC derivatized amino acids including 19 protein and 8 non-protein amino acids were tested, and 19 out of them were enantiomerically discriminated obtaining baseline separation for 11 of them. Analytical characteristics of the method were evaluated for norvaline and tryptophan in terms of linearity, precision, accuracy, limits of detection (LOD) and quantitation (LOQ) showing good performance to be applied to the enantiomeric determination of these amino acids in dietary supplements. LOD and LOQ values were 9.3 and 31 μM for norvaline enantiomers and 7.5 and 25 μM for tryptophan enantiomers, respectively. The contents of D-norvaline and D-tryptophan were below their respective LODs in all the analyzed samples. Quantitation of L-tryptophan and L-norvaline showed good agreement with the labeled contents except for one sample which did not show presence of L-norvaline, contrary to the label indication.

Keywords:

9-Fluorenylmethoxycarbonyl; Amino acids; Chiral separation; Nano-LC; Quinidine-silica hybrid monolithic column

1. Introduction

Amino acids are essential and ubiquitous compounds playing a vital role in many living organisms. Differences in the properties of D- and L-enantiomers have been widely reported, both for protein and non-protein amino acids [1]. For instance, the L-enantiomer of the non-protein amino acid norvaline enhances the nitric oxide production, which is an important regulator and mediator in physiological and pathophysiological events such as vasodilatation [2]. Moreover, it can be used to treat artificial metabolic syndrome in a rat model [3], and it has been proven to be effective in Alzheimer's disease (AD) [4, 5]. However, to the best of our knowledge, D-norvaline has not been reported as an effective substance. On the other hand, L-tryptophan is a protein amino acid which is the precursor of important neurotransmitters, hormones and other relevant biomolecules [6]. The level of L-tryptophan in humans has been reported to act as a biomarker of certain diseases [7, 8]. At the pharmacological level, L-tryptophan is used as antidepressant agent [9], whereas that D-tryptophan is considered as an impurity because of its low biological activity [10, 11]. In addition, only the L-enantiomer of tryptophan is involved in the synthesis of proteins and, additionally, it can cross the blood-brain, being the precursor of the important neurotransmitter serotonin [12]. Amino acids can be used as ingredients in food supplements where the presence of the D-enantiomer is not allowed by legal regulations [13]. Therefore, the enantiomeric determination of DL-amino acids in real samples remains as a very important challenge.

In order to obtain satisfactory chiral separation of amino acids, various chiral stationary phases (CSPs) have been developed, including those based on cyclodextrins [14], polysaccharides [15], 3,5-dinitrobenzamide-naphthylglycine derivatives [16], macrocyclic antibiotics [17] and cinchona alkaloids [18-21]. Cinchona alkaloids, especially quinine and quinidine have attracted much attention owing to their excellent enantioselectivity for amino acids. Usually, because of the low detection sensitivity and weak interaction between the chiral selector and analytes, different derivatization groups were used to react with the amino acids, such as 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [22-24], 9-fluorenylmethoxycarbonyl (FMOC) fluoride [25, 26], 2,4-dinitrofluorobenzene (DNFB) [27], dansyl chloride (DNS-Cl) [28], and carbazole-9-carbonyl chloride (CC-Cl) [18]. Among the different derivatization reagents, FMOC not only allows the amino acids to achieve satisfactory enantioresolution, but also has the advantage of reacting with primary and secondary

amines in just 3 min.

Over the past several decades, monolithic columns have exhibited high separation efficiency and sensitivity, low sample and elution solvent consumption, as well as the ease of coupling to MS [29-32]. So far, the use of quinine or quinidine functionalized monolithic columns has been reported in the enantioseparation of amino acids in capillary electrochromatography (CEC) and nano-liquid chromatography (nano-LC). For instance, Lämmerhofer *et al.* [18, 33] separated some N-derivatized amino acid standards with high efficiency and enantioresolution on the quinine and quinidine silica-based monolithic column by CEC. On the other hand, Wang *et al.* [25, 29] developed several quinidine polymer-based monolithic columns which were also successful to enantioseparate N-derivatized amino acid standards by nano-LC. However, on the one hand, the silica or polymer based monolithic column was limited by the cumbersome and multi-step preparation method, or low mechanical strength and poor stability, on the other hand, they focused on the development of the novel monolithic columns, but their application in the real sample was not reported.

Silica-hybrid monolithic columns offer great advantages such as high surface, little shrinkage, excellent pH stability and good permeability [34-36]. However, they have scarcely been employed for the enantioseparation of amino acids. Thus, Tran *et al.* [19] only separated dinitrobenzoyl-leucine on the quinidine-silica/zirconia hybrid monolithic column by CEC and Kato *et al.* [37] tried to enantioseparate DL-tryptophan on a new-type bovine serum albumin (BSA)-encapsulated hybrid monolithic column but unfortunately, the enantioresolution was very limited. In a previous work, our research group prepared an O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine (MQD)-silica hybrid monolithic column and the enantiomeric separation of protein and non-protein amino acids was investigated using up to 8 different derivatization reagents including 3,5-dinitrobenzoyl chloride, 3,5-dichlorobenzoyl chloride, *p*-nitrobenzoyl chloride, 3,5-dimethoxybenzoyl chloride, *m*-chlorobenzoyl chloride, *p*-chlorobenzoyl chloride, benzoyl chloride, and FMOC chloride [38]. Nevertheless, from the 8 FMOC-amino acids tested, only 2 of them were baseline separated in the reversed phase mode (valine (Rs 1.55) and isoleucine (Rs 1.71)) and none of them was baseline separated in the polar organic phase mode [38]. Moreover, the applicability of quinidine silica-hybrid monolithic columns to the quantitative analysis of amino acids in real samples has not been demonstrated yet.

In this work, the enantiomeric separation of protein and non-protein FMOC

derivatized amino acids was achieved by nano-LC using the above-mentioned MQD-silica hybrid monolithic column. In order to obtain satisfactory enantioresolution performance in the reversed phased mode, the mobile phase was systematically optimized, including the apparent pH, content of ACN, and buffer concentration. Under the optimized conditions, 27 FMOC-derivatized amino acids consisting of 19 protein and 8 non-protein amino acids were tested. Finally, analytical characteristics of the developed method were evaluated for norvaline and tryptophan and the method was applied to the quantitation of L-norvaline and L-tryptophan in dietary supplements.

2. Materials and methods

2.1. Reagents and ssmples

All reagents were of analytical grade. Methanol (MeOH), acetonitrile (ACN), and acetic acid (HAc) were acquired from Scharlau Chemie (Barcelona, Spain). Triethylamine (TEA) and 9-fluorenylmethoxycarbonyl (FMOC) chloride were obtained from Fluka (Buchs, Switzerland). Ammonium hydroxide ($NH_3 \cdot H_2O$), boric acid (H_3BO_3) and pentane were from Sigma (St. Louis, Missouri, USA), and ammonium acetate was from Merck (Darmstadt, Germany). 10,11-dihydroquinidine, 2,2'-azobisisobutyronitrile (AIBN), 3-(trimethoxysilyl)-propylmethacrylate (γ-MAPS), vinyltrimethoxysilane (VTMS), cetyltrimethylammonium bromide (CTAB), tetramethoxysilane (TMOS) and ethylene glycol (EG) were acquired from Aladdin Chemicals (Shanghai, China). DL-arginine, DL-histidine, DL-lysine, DL-serine, DL-threonine, DL-asparagine, DL-glutamine, DL-cysteine, DL-proline, DL-alanine, DL-valine, DL-leucine, DL-methionine, DL-phenylalanine, DL-tyrosine, D-tryptophan, L-tryptophan, DL-ornithine, DL-citrulline standards were from Fluka (Buchs, Switzerland), while DL-isoleucine, DL-carnitine, DL-aspartic acid, DL-glutamic acid, DL-norvaline, L-norvaline, DL-norleucine, DL-DOPA, DL-pyroglutamic acid and DL-methionine sulfone were obtained from Sigma (St. Louis, Missouri, USA). FMOC-amino acids were synthesized as reported previously [29, 30]. The dietary supplements were obtained in capsule form from online sources.

2.2. Instrumentation

All nano-LC experiments were conducted on a laboratory self-assembled nano-LC instrument. The system consists of a Shimadzu LC-20AD pump (Kyoto, Japan), a Linear Instruments UV-Vis 200 detector (California, USA), and a Valco four-port

injection valve with 20 nL internal loop (Houston, USA). In order to reduce the flow and pressure, a stainless-steel tee (Cheminert, Valco Instruments Houston, Texas, USA) with flow split capillary (150 mm × 25 μm I.D.) was employed before the injection valve. The data acquisition and data handling were performed using the software Chromatostation N200 (Zhejiang University, China). All chromatograms were converted to a text file and redrawn using Microcal Origin 8.5. pH values of buffer solutions were measured in a 744 pH meter (Herisau, Switzerland).

2.3. Chromatographic conditions

Mobile phase was prepared by mixing the 3 mM ammonium acetate with ACN (35/65, v/v), and adjusting the apparent pH to the desired value (pH 4.8) with acetic acid. At the beginning, the mobile phase was subjected to filtration through a 0.22 μm membrane and sonication degas prior to be employed. The total flow rate was 10 μL/min, total backpressure was 23 bar, injection volume was 20 nL, and the UV detection wavelength was 254 nm.

2.4. Preparation of the MQD-silica hybrid monolithic column

Synthesis of the MQD-silica hybrid monolithic column was conducted as previously reported [38]. Briefly, anchoring sites for the bulk polymer on the inner capillary walls were generated using γ-MAPS/MeOH (50/50, v/v). The pre-polymerizable mix for the MQD column was obtained as follows: MQD (6.0 mg), MeOH (100 μL), EG (30 μL), CTAB (1.6 mg), H_2O (30 μL), $NH_3 \cdot H_2O$ (0.02 M, 30 μL), TMOS (60 μL), VTMS (80 μL), and AIBN (1 mg) were mixed in a 2-mL vial and sonicated during 5 min at room temperature. The obtained homogeneous solution was introduced into the 30 cm pre-treated capillary. Both ends of the capillary column were sealed using GC septa and capillary was placed in a water bath at 40 ºC during 12 h first, then at 60 ºC for another 12 h. Non-reacted CTAB and other waste products were rinsed out by flushing the column with MeOH. The monolithic column was cut to 15 cm for further use.

2.5. Preparation of the standard and dietary supplements solutions

FMOC-amino acids standard solutions were prepared by dissolving the corresponding amino acid standards into 1 mL of 200 mM H_3BO_3 (pH 9.0) to obtain a 10 mM solution, and were sonicated for 2 min. Then, 1 mL of a 220 mM FMOC-Cl solution in acetonitrile was added and it was let to react at room temperature for 3 min.

Afterwards, 2 mL of pentane were added into the solution and it was vortexed. After 5 min of resting, the lower layer solution was diluted at the desired concentration using the mobile phase. FMOC-dietary supplement solutions were prepared by dissolving the powder within the capsules (200 mg norvaline or 2 mg tryptophan) into 1 mL of 200 mM H_3BO_3 (pH 9.0) and were sonicated for 2 min, then they were filtered and 1 mL of 220 mM FMOC-Cl in acetonitrile was then added into the filtrate. Reaction took place for 3 min at room temperature. Afterwards, 2 mL of pentane were added into the solution and it was vortexed. Finally, after 5 min of resting, the lower layer solution was diluted at the desired concentration using the mobile phase.

3. Results and discussion

3.1. Enantioseparation of FMOC-amino acids by nano-LC using a MQD-silica hybrid monolithic column

The MQD-silica hybrid monolithic column was prepared as detailed in section 2.4. In a previous work from our research group, the enantiomeric separation of protein and non-protein amino acids was investigated using up to 8 different derivatization reagents [38]. However, when using FMOC as derivatizing reagent, from the eight amino acids tested, only two of them were baseline separated in the reversed phase mode (10 mM ammonium acetate/ACN (30/70, v/v) (apparent pH 5.3)) (valine (Rs 1.55) and isoleucine (Rs 1.71)) and none of them was baseline separated in the polar organic phase mode (0.055 % HAc, 0.005% TEA in ACN/MeOH (60/40, v/v)) [38]. As shown in **Fig. S1**, when comparing the polar organic-phase and reversed-phase modes for some FMOC-amino acids, the latter enabled obtaining better peak shapes [38]. Thus, the reversed phase mode was selected in order to obtain a good enantioresolution for the FMOC derivatized amino acids. With this aim, in this work, the mobile phase was optimized using four FMOC derivatized amino acids, i.e. two protein amino acids (methionine and valine) and two non-protein amino acids (methionine sulfone and norvaline).

First, the effect of apparent pH was studied on the retention factors (k_1 and k_2), enantioselectivity (α), enantioresolution (Rs) and efficiency (N_1 and N_2). As shown in **Table 1**, the apparent pH values were evaluated from 4.3 to 5.3 in the mobile phase 10 mM ammonium acetate/ACN (30/70, v/v), while other conditions were kept constant. On the one hand, when increasing the apparent pH value from 4.3 to 5.3, the

enantioselectivity kept constant but the retention factors increased for the four analytes. This can be explained because the CSP is full of positive charges when the pH of the mobile phase is between 4.3 and 5.3, while the four FMOC-derivatized amino acids are negatively charged, hence leading to a stronger electrostatic interaction between the analytes and the CSP. On the other hand, the enantioresolution significantly increased when the apparent pH value changed from 4.3 to 4.8, while the efficiency gradually decreased. Further increasing in the apparent pH value to 5.3 lead to similar values of enantioresolution, while the efficiency decreased significantly. Considering the retention factors, enantioresolution and efficiency, an apparent pH of 4.8 was selected for the following experiments.

Second, the influence of the buffer concentration was investigated while keeping constant the mobile phase apparent pH at 4.8 and composition as ammonium acetate/ACN (30/70, v/v). As shown in **Table 1**, the retention factors, analysis time and enantioresolution values decreased when increasing the concentration of the ammonium acetate buffer from 1 to 10 mM, meanwhile the analysis time and efficiency increased. Comparing the efficiency, analysis time, and enantioresolution obtained in 1 and 3 mM of ammonium acetate buffer, the efficiency was significantly higher when the buffer concentration increased, but the analysis time increased from 35 min to 60 min (such as in the case of methionine and methionine sulfone), whereas that the enantioresolution did not significantly changed. Hence, a buffer concentration of 3 mM was selected as a compromise between the analysis time and efficiency.

Third, the ACN content was investigated by varying its percentage from 60 to 80%. As can be seen in **Table 1**, the retention factor and enantioresolution decreased when increasing the ACN content from 60 to 80%, while apparent pH=4.8 and 3 mM ammonium acetate were kept constant. The hydrophobic interaction between the FMOC derivatized amino acids and MQD-silica hybrid monolithic column is weakened when the organic solvent content in the mobile phase is increased because the elution ability of the mobile phase increases. Considering the analysis time, enantioresolution and efficiency, 65% ACN was selected as the optimum condition in the mobile phase.

Finally, using a 3 mM ammonium acetate/ACN (35/65, v/v) (apparent pH=4.8) mobile phase, 27 FMOC-labelled amino acids (19 proteins and 8 non-proteins) were assayed. Chiral discrimination for 19 amino acids was observed and a total of 11 FMOC-derivatized amino acids (9 proteins and 3 non-proteins) were baseline or almost baseline enantioseparated (Rs 1.46 was obtained for tryptophan) (see **Table S1**). **Fig. 1**

displays the chiral separation of some representative amino acids.

Table 1. Effect of mobile phase composition and pH on the enantioseparation data for FMOC derivatized amino acids.

	Sample	k_1	k_2	α	R_s	N_1 (m)	N_2 (m)
Effect of the apparent pH[a]							
pH=4.3	FMOC-methionine	0.83	1.00	1.21	0.73	14700	13600
	FMOC-valine	0.52	0.69	1.33	0.93	17200	17000
	FMOC-methionine sulfone	0.88	1.08	1.22	0.76	14000	12000
	FMOC-norvaline	0.53	0.64	1.20	0.54	14900	12800
pH=4.8	FMOC-methionine	1.65	2.00	1.21	1.20	14200	13000
	FMOC-valine	1.11	1.47	1.33	1.45	14500	14300
	FMOC-methionine sulfone	1.54	1.90	1.23	1.10	11900	11500
	FMOC-norvaline	1.13	1.37	1.21	0.97	14700	14200
pH=5.3	FMOC-methionine	3.42	4.06	1.19	0.84	13200	8900
	FMOC-valine	2.57	3.35	1.30	1.55	12000	9700
	FMOC-methionine sulfone	1.91	2.33	1.22	0.81	13400	8500
	FMOC-norvaline	2.56	3.14	1.23	0.88	11000	8200
Effect of the buffer concentration[b]							
1.0 mM	FMOC-methionine	7.85	9.52	1.21	1.58	7500	6000
	FMOC-valine	5.25	7.12	1.36	1.87	5600	4600
	FMOC-methionine sulfone	8.01	10.16	1.27	1.46	5800	4800
	FMOC-norvaline	5.77	7.09	1.23	1.46	5900	4600
3.0 mM	FMOC-methionine	4.26	5.15	1.21	1.52	13200	12700
	FMOC-valine	2.82	3.78	1.34	1.74	12500	11800

	FMOC-methionine sulfone	4.06	4.99	1.23	1.46	10100	9600
	FMOC-norvaline	2.85	3.48	1.22	1.44	12500	11200
	FMOC-methionine	2.59	3.16	1.22	1.44	13600	13100
5.0 mM	FMOC-valine	1.75	2.32	1.33	1.56	13500	12800
	FMOC-methionine sulfone	2.58	3.20	1.24	1.44	12000	11000
	FMOC-norvaline	1.89	2.28	1.21	1.37	15800	15000
	FMOC-methionine	1.65	2.00	1.21	1.20	14200	13000
	FMOC-valine	1.11	1.47	1.33	1.45	14500	14300
10 mM	FMOC-methionine sulfone	1.54	1.90	1.23	1.10	11900	11500
	FMOC-norvaline	1.13	1.37	1.21	0.97	14900	14200
Effect of ACN content[c]							
	FMOC-methionine	8.78	10.75	1.23	1.61	12500	11000
	FMOC-valine	6.07	8.05	1.33	1.95	12100	11800
60%	FMOC-methionine sulfone	6.46	7.91	1.23	1.54	12300	11000
	FMOC-norvaline	6.24	7.58	1.21	1.52	11700	10200
	FMOC-methionine	6.11	7.40	1.21	1.58	13000	11800
	FMOC-valine	4.13	5.54	1.34	1.92	12400	11500
65%	FMOC-methionine sulfone	4.95	6.07	1.23	1.52	8400	7100
	FMOC-norvaline	4.26	5.25	1.23	1.52	14400	11500
	FMOC-methionine	4.26	5.15	1.21	1.52	13200	12700
	FMOC-valine	2.82	3.78	1.34	1.74	12500	11800
70%	FMOC-methionine sulfone	4.06	4.99	1.23	1.46	10100	9600
	FMOC-norvaline	2.85	3.48	1.22	1.44	12500	11200
75%	FMOC-methionine	2.98	3.60	1.21	1.45	13600	13300

	FMOC-valine	1.99	2.63	1.32	1.59	12700	11700
	FMOC-methionine sulfone	3.27	4.01	1.23	1.39	11700	10100
	FMOC-norvaline	1.99	2.41	1.22	1.41	12500	11100
	FMOC-methionine	1.89	2.27	1.20	1.24	15800	15100
80%	FMOC-valine	1.21	1.60	1.32	1.51	15400	14200
	FMOC-methionine sulfone	2.48	3.01	1.21	1.29	13200	12800
	FMOC-norvaline	1.21	1.46	1.21	1.01	16100	15800

Conditions: (a) 10 mM ammonium acetate/ACN (30/70, v/v) (at the desired apparent pH values); (b) ammonium acetate/ACN (at the desired concentration of the buffer) (30/70, v/v) (apparent pH=4.8); (c) 3 mM ammonium acetate/ACN (at the desired ratio of ACN and buffer, v/v) (apparent pH=4.8). Other experimental conditions as in **Fig. 1**.

3.2. Application of the developed method to the enantiomeric determination of tryptophan and norvaline in dietary supplements

Based on the interest of tryptophan and norvaline as ingredients in dietary supplements, the developed method was applied to the enantiomeric determination of these two amino acids in different dietary supplements. With this aim, the analytical characteristics of the method were evaluated in terms of selectivity, linearity, precision, accuracy and limits of detection (LOD) and quantitation (LOQ) both for norvaline (**Table 2**) and for tryptophan (**Table 3**). The selectivity was suitable for tryptophan and norvaline enantiomers because they were separated and there were no interfering peaks which originated from the sample matrices. The linearity results were obtained from six standard solutions at different concentration levels, injected in triplicate. As shown in **Tables 2** and **3**, the linearity for the two analytes was demonstrated to be adequate in all cases as R^2 values were $\geq$ 99.0%, and confidence intervals for the slope did not include the zero value, meanwhile confidence interval for the intercept included the zero value for a 95% confidence level.

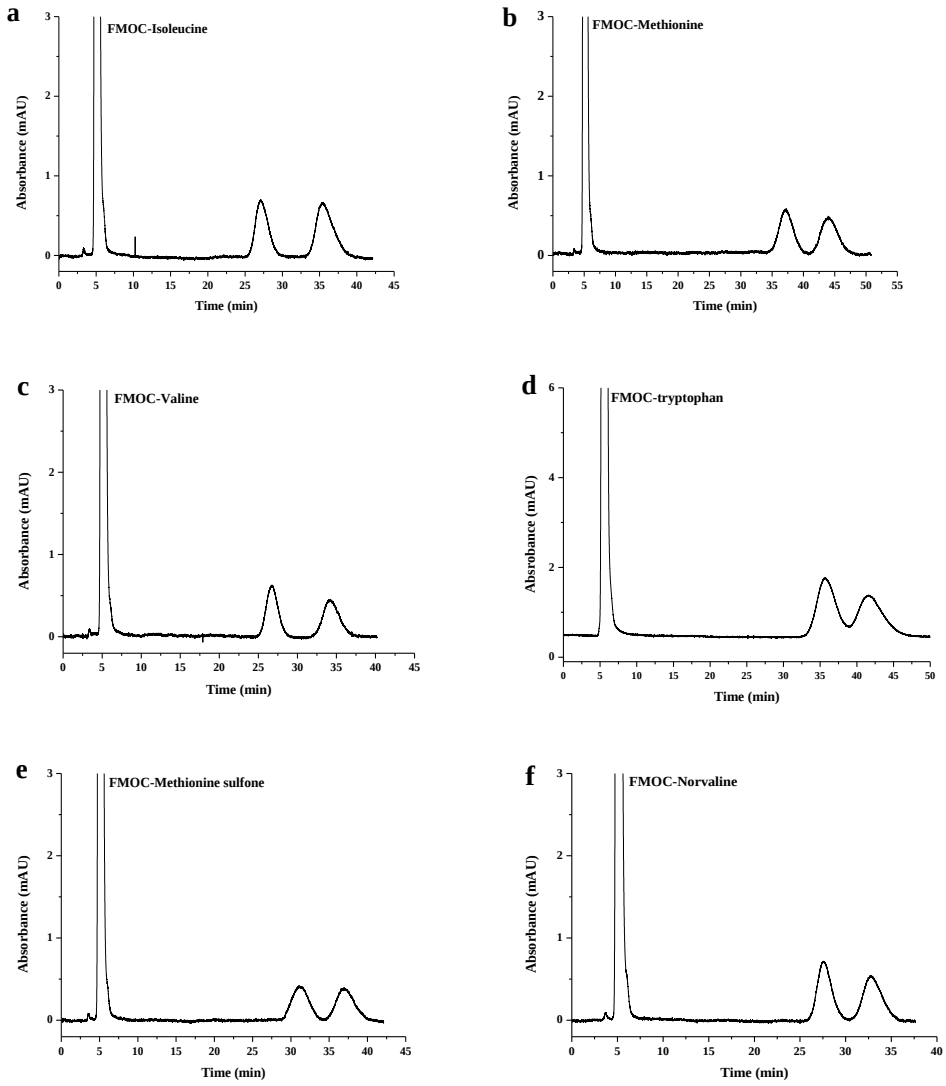


Fig. 1. Enantioseparation of some FMOC-derivatized amino acids. Experimental conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: ACN/3 mM ammonium acetate (65/35, v/v) (apparent pH=4.8); UV detection wavelength: 254 nm; flow rate: 10 μL/min; backpressure: 23 bar; injection volume: 20 nL.

In order to assess the presence of matrix interferences, slopes of the calibration curves obtained from the external standard and standard additions calibration methods were compared using analysis of variance (ANOVA) test. Both for tryptophan and norvaline, the p-values of ANOVA were above 0.05 which means that there were no matrix interferences for a 95% confidence level. Therefore, the external standard calibrations method was considered appropriate to perform the quantitation of the analytes in the dietary supplements. Accuracy was evaluated as the recovery obtained from both spiked solutions of norvaline and tryptophan at different labeled content percentages (80, 100 and 120%) injected in triplicate.

Table 2. Analytical characteristics of the developed method for the enantiomeric determination of norvaline in dietary supplements.

	L-norvaline First enantiomer	D-norvaline Second enantiomer
External standard calibration method[a]		
Range	0.031-12.5 mM	0.031-1.25 mM
Slope±t·S_{slope}	479786±5243	478792±7646
Intercept±t·$S_{intercept}$	20902±24051	-363±4295
R^2	99.93%	99.91%
Standard addition calibration method[b]		
Study of matrix interferences (p-value of ANOVA)	0.625	0.779
Accuracy[c]		
Mean recovery (%)	109±3%	105±6%
Precision		
Instrumental repeatability[d]		
t, RSD (%)	0.45	0.38
A, RSD (%)	0.91	1.10
Method repeatability[e]		
t, RSD (%)	1.01	1.14
A, RSD (%)	4.86	5.74
Intermediate precision[f]		
t, RSD (%)	2.14	2.03
A, RSD (%)	5.68	5.45
LOD[g]	9.3 μM	
LOQ[h]	31 μM	

(a) Six standard solutions at different concentration levels injected in triplicate (Concentration of L-norvaline: 12.5, 5.0, 2.5, 1.25, 0.5 and 0.25 mM; concentration of D-norvaline: 1.25, 0.5, 0.25, 0.125, 0.05, and 0.025 mM).
(b) Addition of six known amounts of D- and L-norvaline (Concentration of added L-norvaline: 0.167, 0.250, 0.333, 0.417, 0.500, and 0.583 mM; concentration of added D-norvaline: 0.033, 0.050, 0.067, 0.083, 0.100, and 0.117 mM) to a dietary supplement sample 1 containing a constant concentration of L-norvaline.
(c) Accuracy was evaluated as the recovery obtained from three dietary supplements spiked with standard D, L-norvaline at three different percentages (80, 100, and 120 %) of the labeled content (n = 3).
(d) Consecutive injections of a D, L-norvaline standard solution (0.125 mM) (n = 6).
(e) 200 mg dietary supplement sample 1 containing 1 mg of L-norvaline (as labeled amount) spiked with 2 mg of L and 2 mg of D-norvaline (n = 6).
(f) 200 mg dietary supplement sample 1 containing 1 mg of L-norvaline (as labeled amount) spiked with 2 mg of L and 2 mg of D-norvaline in three days (n = 9).
(g) LOD obtained for a S/N equal to 3.
(h) LOQ obtained for a S/N equal to 10.
Experimental conditions as in **Fig. 1**.

As shown in **Tables 2** and **3**, recovery values for the two analytes were satisfactory as all of them were near the 100%. Precision was evaluated as the instrumental repeatability, method repeatability and intermediate precision. As **Tables 2** and **3** show, regarding the instrumental repeatability, the RSD values (%) for areas and retention times were lower than 1 and 2%, respectively. For the method repeatability, the RSD values (%) for areas and retention times were also adequate, with values lower than 2 and 6%, respectively. Moreover, in the case of intermediate precision, the RSD values (%) were lower than 10% for both the areas and retention times.

Table 3. Analytical characteristics of the developed method for the enantiomeric determination of tryptophan in dietary supplements.

	L-tryptophan First enantiomer	D-trytophan Second enantiomer
External standard calibration method[a]		
Range	0.025-12.5 mM	0.025-1.25 mM
Slope±t·S_{slope}	599205±1696	598197±16093
Intercept±t·$S_{intercept}$	7267±7782	3259±9041
R^2	99.95%	99.96%
Standard addition calibration method[b]		
Study of matrix interferences (p-value of ANOVA)	0.821	0.887
Accuracy[c]		
Mean recovery (%)	98±2%	101±3%
Precision		
Instrumental repeatability[d]		
t, RSD (%)	0.48	0.56
A, RSD (%)	0.50	1.11
Method repeatability[e]		
t, RSD (%)	1.10	1.30
A, RSD (%)	4.71	4.00
Intermediate precision[f]		
t, RSD (%)	4.15	4.44
A, RSD (%)	6.15	6.69
LOD[g]	7.5 μM	
LOQ[h]	25 μM	

(a) Six standard solutions at different concentration levels injected in triplicate (Concentration of L-

and D-tryptophan same as norvaline (**Table 2**)).

(b) Addition of six known amounts of D- and L-tryptophan (Concentration of added L-tryptophan: 0.139, 0.167, 0.208, 0.278, 0.417, and 0.833 mM; concentration of added D-tryptophan: 0.028, 0.033, 0.042, 0.056, 0.083, and 0.167 mM) to a dietary supplement sample 1 containing a constant concentration of L-tryptophan.

(c) Accuracy was evaluated as in **Table 2**.

(d) Consecutive injections of a D, L-tryptophan standard solution (0.333 mM) (n = 6).

(e) 2 mg dietary supplement sample 1 containing 2 mg of L-tryptophan (as labeled amount) spiked with 2 mg of D-tryptophan (n = 6).

(f) 2 mg dietary supplement sample 1 containing 2 mg of L-tryptophan (as labeled amount) spiked with 2 mg of D-tryptophan in three days (n = 9).

(g) LOD obtained for a S/N equal to 3.

(h) LOQ obtained for a S/N equal to 10.

Experimental conditions as in **Fig. 1**.

Regarding LOD and LOQ values, as it can be seen in **Tables 2** and **3**, these values were 9.3 and 31 μM for norvaline enantiomers, and 7.5 and 25 μM for tryptophan enantiomers. **Fig. 2a** and **2b**, and **3a** and **3b** display the chromatograms corresponding to the enantiomeric separation of norvaline and tryptophan, respectively, at concentrations corresponding to their LOD and LOQ.

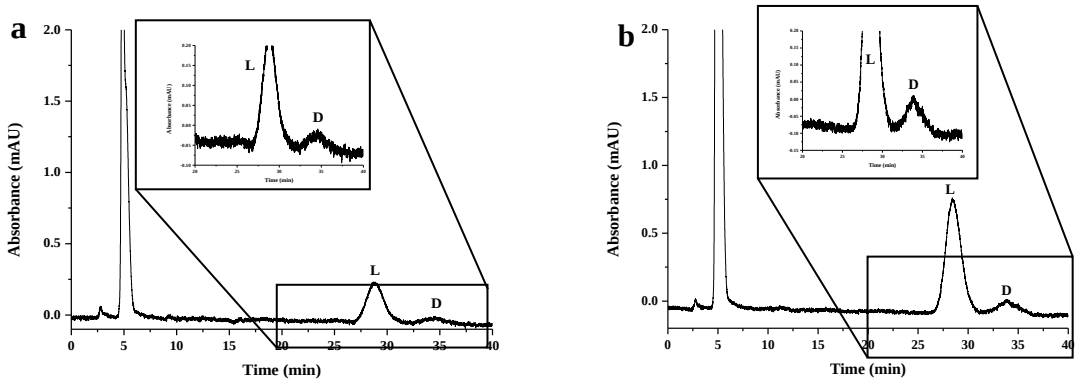


Fig. 2. Chromatograms corresponding to (a) the LOD (9.3 µM) and (b) the LOQ (31 µM) of D-norvaline. Experimental conditions: 15 cm × 100 µm I.D.; mobile phase: ACN/3 mM ammonium acetate (65/35, v/v) (apparent pH=4.8); UV detection wavelength: 254 nm; flow rate: 10 µL/min; backpressure: 23 bar; injection volume: 20 nL.

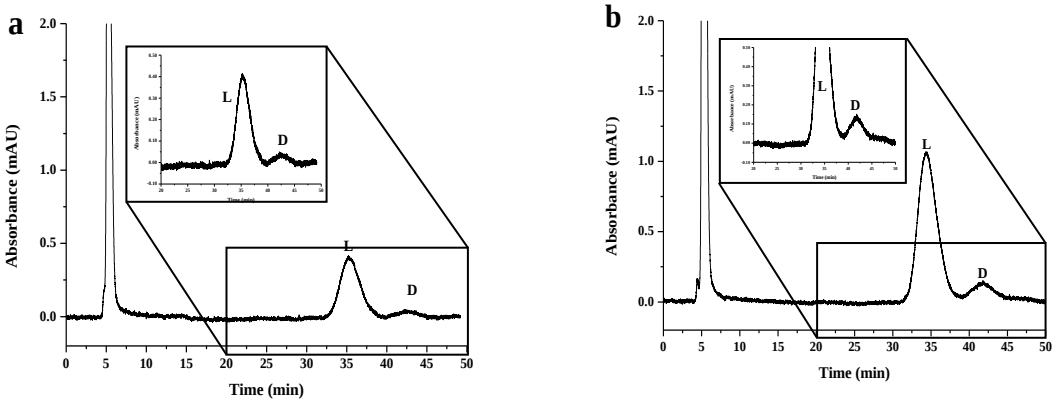


Fig. 3. Chromatograms corresponding to (a) the LOD (7.5 µM) and (b) the LOQ (25 µM) of D-tryptophan. Experimental conditions: 15 cm × 100 µm I.D.; mobile phase: ACN/3 mM ammonium acetate (65/35, v/v) (apparent pH=4.8); UV detection wavelength: 254 nm; flow rate: 10 µL/min; backpressure: 23 bar; injection volume: 20 nL.

Once the developed method was proven to be adequate for the enantiomeric determination of tryptophan and norvaline, it was applied to the analysis of four dietary supplements containing L-tryptophan and two containing L-norvaline. As it can be seen in **Fig. 2c**, D-norvaline content was below the LOD (9.3 µM) in samples 1 and 2, while the content of L-norvaline in sample 2 was above the LOQ value and the amount quantified by the standard external calibration method was in agreement with the labeled content (**Table 4**). Regarding sample 1, content of L-norvaline did not match the labeled content, since it was below the LOD. Preconcentrating 4 times sample 1 still lead to no detection of L-norvaline. Spiking sample 1 with 125 µM of L-norvaline

resulted in the detection of this peak being the calculated concentration equal to the added one. A second batch of sample 1 was also injected (data not shown) and the outcome was the same as with the first batch, L-norvaline could not be detected. Consequently, it could be concluded that L-norvaline was not present in this sample in spite of the fact that the label declared its presence.

As in the case of L-norvaline dietary supplements, the content of D-tryptophan in the four supplements analyzed was below the LOD value (**Fig. 3c**). Content of L-tryptophan was in agreement with the label in all cases (**Table 4**).

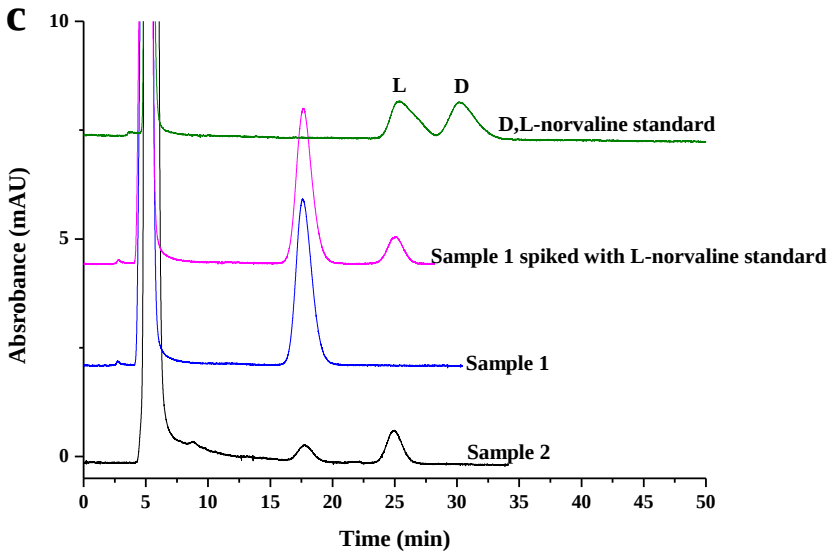


Fig. 2c. Chromatograms corresponding to FMOC-derivatized racemic norvaline standard solution and analyzed dietary supplements whose label indicated presence of L-norvaline (samples 1 and 2). Experimental conditions: 15 cm × 100 μm I.D.; mobile phase: ACN/3 mM ammonium acetate (65/35, v/v) (apparent pH=4.8); UV detection wavelength: 254 nm; flow rate: 10 μL/min; backpressure: 23 bar; injection volume: 20 nL.

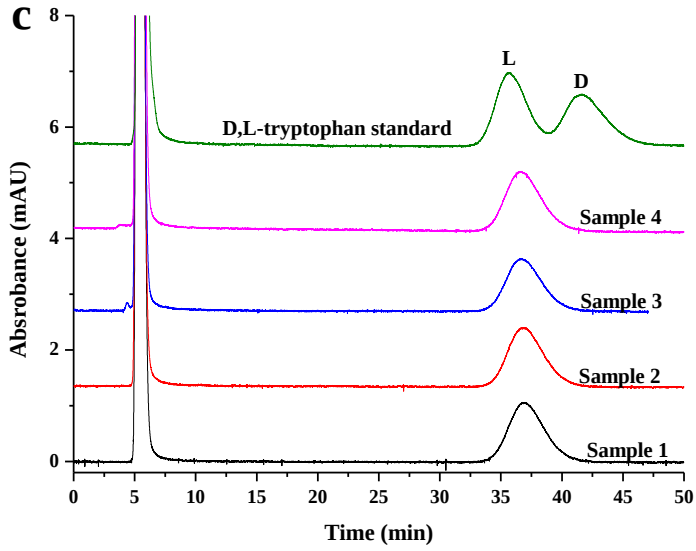


Fig. 3c. Chromatograms corresponding to FMOC-derivatized racemic tryptophan standard solution and analyzed dietary supplements whose label indicates presence of L-tryptophan (samples 1-4). Experimental conditions: 15 cm × 100 μm I.D.; mobile phase: ACN/3 mM ammonium acetate (65/35, v/v) (apparent pH=4.8); UV detection wavelength: 254 nm; flow rate: 10 μL/min; backpressure: 23 bar; injection volume: 20 nL.

Table 4. Results obtained in the analysis of norvaline and tryptophan in dietary supplements.

Sample	D- enantiomer content (%)	L- enantiomer content (%)
Norvalin-sample 1	<LOD	<LOD
Norvalin-sample 2	<LOD	104±4.0
Tryptophan-sample 1	<LOD	102.8±4.1
Tryptophan-sample 2	<LOD	97.2±2.9
Tryptophan-sample 3	<LOD	109.6±3.6
Tryptophan-sample 4	<LOD	106.4±2.3

Experimental conditions as in **Fig. 1.**

4. Conclusions

The enantiomeric separation of FMOC-derivatized protein and non-protein amino acids was achieved by nano-LC using a MQD-silica hybrid monolithic column. First, the FMOC was selected as the derivatization reagent which has the simplest sample preparation and satisfactory enantioresolution. Second, the effect of the apparent pH,

buffer concentration and content of the ACN in the mobile phase was investigated for a group of FMOC-derivatized amino acids. Third, under the optimized conditions, the MQD-silica hybrid monolithic column offered good enantiorecognition for 19 analytes, enabling baseline enantioseparation for 11 out of 27 assayed FMOC-amino acids. Finally, the method was proven to be adequate in terms of selectivity, linearity, precision, accuracy and LODs and LOQs for the enantiomeric determination of L-norvaline and L-tryptophan in dietary supplements. From the analyzed dietary samples, none of them presented detectable amounts of the D-enantiomers while the content of the L-enantiomers was in agreement with the label content in all cases, except for one L-norvaline sample whose presence was completely missing although the label indicated its presence. This demonstrates the applicability of the developed strategy in the quality control of dietary supplements in a fast and accurate manner. It is also the first report showing the application of the MQD silica-hybrid monolithic column to analysis of real samples.

Appendix D.

Supplemental material for article 4.

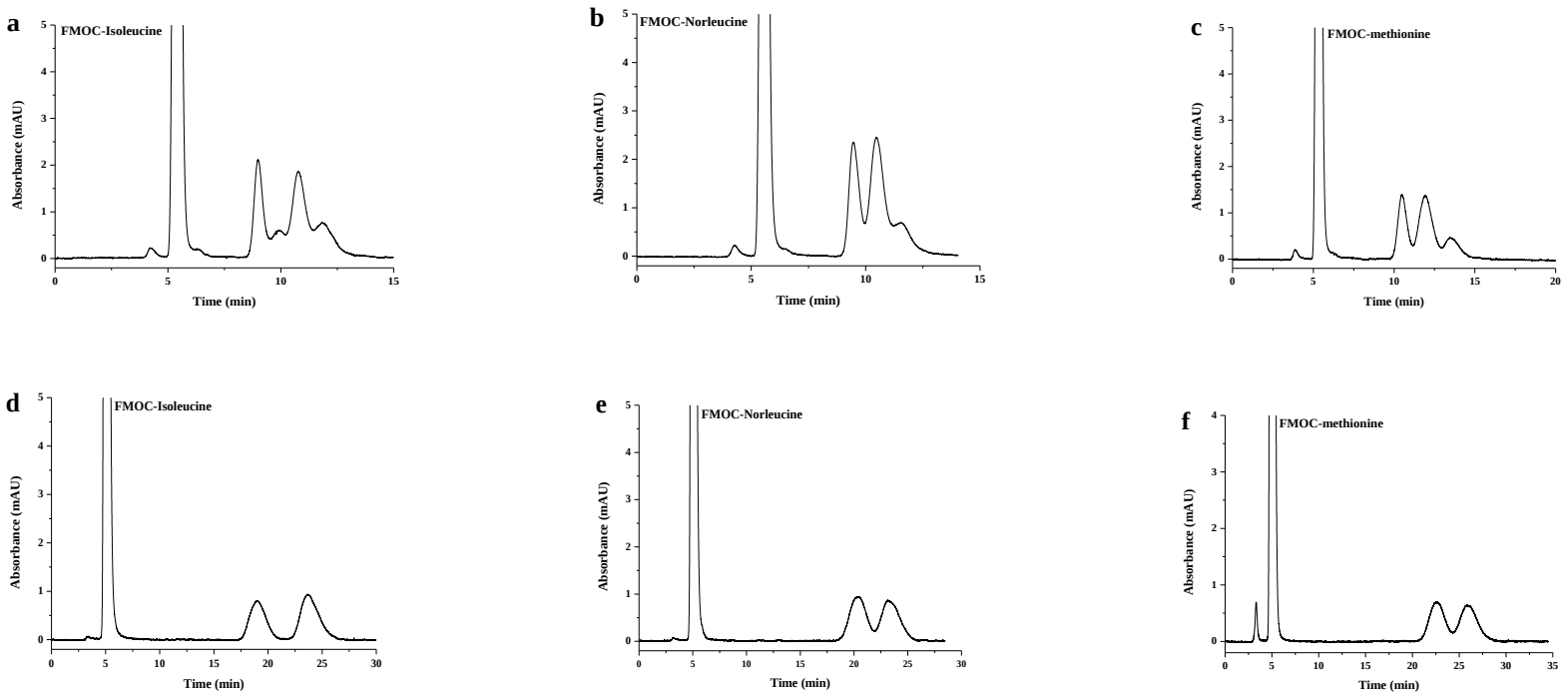


Fig. S1. Comparison of the enantiomeric separation of three FMOC-derivatized amino acids under the polar organic phase mode (a, b and c) and the reversed phase mode (d, e, and f). Experimental conditions: column dimensions: 15 cm × 100 μm I.D.; polar organic phase mode: ACN/MeOH/HAc/TEA (60/40/0.055/0.005, v/v/v/v); and reversed phase mode: 10 mM ammonium acetate/ACN (30/70, v/v) (apparent pH=5.3); UV detection wavelength: 254 nm; flow rate: 10 μL/min; injection volume: 20 nL.

Table S1. Enantioseparation data for FMOC derivatized amino acids under the optimized conditions.

Sample	k_1	k_2	α	R_s	N_1 (m)	N_2 (m)
FMOC-protein amino acids						
Serine	4.51	5.73	1.27	1.64	12500	11100
Isoleucine	4.21	5.82	1.38	2.14	12800	10300
Methionine	6.11	7.40	1.21	1.58	13000	11800
Valine	4.13	5.54	1.34	1.92	12400	11500
Alanine	3.92	4.46	1.14	1.23	18200	16300
Tryptophan	6.09	7.37	1.21	1.46	12000	11000
Histidine	12.29	15.10	1.23	1.58	17000	12000
Lysine	8.03	9.23	1.15	0.93	12000	10100
Threonine	4.21	5.58	1.32	1.68	6800	5400
Arginine			/			
Glutamic acid			/			
Proline			/			
Cysteine	11.39	14.39	1.26	1.78	6500	4300
Asparagine	3.26	3.83	1.17	0.32	6000	5100
Leucine	3.53	4.25	1.20	1.39	7000	6300
Aspartic acid			/			
Glutamine	3.05	3.64	1.19	0.69	8600	7100
Phenylalanine	8.64	10.48	1.21	1.61	11000	10000
Tyrosine	5.89	7.24	1.23	1.42	5100	4000
FMOC-non-protein amino acids						
Norleucine	4.78	5.81	1.22	1.53	6500	4200

Ornithine	9.51	10.85	1.14	0.94	12800	11400
Methionine sulfone	4.95	6.07	1.23	1.52	8400	7100
Norvaline	4.26	5.25	1.23	1.52	14400	11500
Citrulline	/					
DOPA	/					
Carnitine	/					
Pyroglutamic acid	/					

Experimental conditions as in **Fig. 1**. "/" indicates that the analytes could not be separated.

Acknowledgements

M. L. Marina and E. Sánchez-López thank the Ministry of Economy and Competitiveness (Spain) for research projects CTQ2013-48740-P and CTQ2016-76368-P. They also thank the Comunidad of Madrid (Spain) and European funding from FEDER program for research project S2018/BAA-4393 (AVANSECAL-II-CM). D. Xu and E. Sánchez-López thank the Comunidad of Madrid (Spain) and European funding from FEDER program (S2013/ABI-3028, AVANSECAL-CM) for their contracts.

References

[1] L.P. Zhao, J. Qiao, K. Zhang, et al., Construction of chiral ligand exchange capillary electrochromatography for D,L-amino acids enantioseparation and its application in glutaminase kinetics study, J. Chromatogr. A 1548 (2018) 104-110. https://doi.org/10.1016/j.chroma.2018.03.031

[2] C.I. Chang, J.C. Liao, L. Kuo, Arginase modulates nitric oxide production in activated macrophages, Am. Physiol. Soci. 274 (1998) 342-348. https://doi.org/10.1152/ajpheart.1998.274.1.H342

[3] H.M. El-Bassossy, R. El-Fawal, A. Fahmy, et al., Arginase inhibition alleviates hypertension in the metabolic syndrome, Br. J. Pharmacol. 169 (2013) 693-703. https://doi.org/10.1111/bph.12144

[4] X.F. Ming, A.G. Rajapakse, J.M. Carvas, et al., Inhibition of S6K1 accounts partially for the anti-inflammatory effects of the arginase inhibitor L-norvaline, Bmc Cardiovasc Disor. 9 (2009) 9, 1-7. https://doi:10.1186/1471-2261-9-12

[5] B. Polis, K.D. Srikanth, E. Elliott, et al., L-Norvaline Reverses Cognitive Decline and Synaptic Loss in a Murine Model of Alzheimer's Disease, Neurotherapeutics 15 (2018) 1036-1054. https://doi.org/10.1007/s13311-018-0669-5

[6] X.F. Tang, Y. Liu, H.Q. Hou, et al., Electrochemical determination of L-Tryptophan, L-Tyrosine and L-Cysteine using electrospun carbon nanofibers modified electrode, Talanta 80 (2010) 2182-2186. https://doi.org/10.1016/j.talanta.2009.11.027

[7] A. Agazzi, F. De Ponti, R. De Giorgio, et al., Review of the implications of dietary tryptophan intake in patients with irritable bowel syndrome and psychiatric disorders, Digest. Liver Dis. 35 (2003) 590-595. https://doi.org/10.1016/S1590-8658(03)00277-9

[8] J.B. Raoof, R. Ojani, H. Karimi-Maleh, Carbon paste electrode incorporating 1-[4-(ferrocenyl ethynyl) phenyl]-1-ethanone for electrocatalytic and voltammetric

determination of tryptophan, Electroanal. 20 (2008) 1259-1262.
https://doi.org/10.1002/elan.200704176
[9] J. Yang, D.S. Hage, Characterization of the binding and chiral separation of D-and L-tryptophan on a high-performance immobilized human serum albumin column, J. Chromatogr. A 645 (1993) 241-250. https://doi.org/10.1016/0021-9673(93)83383-4
[10] R.R. Langner, C.P. Berg, Metabolism of D-tryptophan in the normal human subject, J. Biol. Chem. 214 (1955) 699-707. http://www.jbc.org/content/214/2/699.
[11] W.C. Rose, G.F. Lambert, M.J. Coon, The amino acid requirements of min. 7. General procedures; the tryptophan requirement, J. Biol. Chem. 211 (1954) 815-827.
http://www.jbc.org/content/211/2/815.citation
[12] D.W. Richard, M.A. Dawes, C.W. Mathias, et al., N. Hill-Kapturczak, D.M. Dougherty, L-tryptophan: basic metabolic functions, behavioral research and therapeutic indications, Int. J. Tryptophan Res. 2 (2009) 45-60.
https://doi.org/10.4137/IJTR.S2129
[13] Commision Decision 2001/15/EC. Off. J. Eur. Commun., 2001, L52, 19-25.
[14] X.B. Yao, T.T.Y. Tan, Y. Wang, Thiol-ene click chemistry derived cationic cyclodextrin chiral stationary phase and its enhanced separation performance in liquid chromatography, J. Chromatogr. A 1326 (2014) 80-88.
https://doi.org/10.1016/j.chroma.2013.12.054
[15] Y.H. Li, C.S. Baek, B.W. Jo, et al., Direct chiral separation of N-fluorenylmethoxycarbonyl α-amino acids by HPLC for determination of enantiomeric purity, Bull. Korean Chem. Soci. 26 (2005) 998-1000.
https://doi.org/10.5012/bkcs.2005.26.6.998
[16] M. Kato, T. Fukushima, N. Shimba, et al., A study of chiral recognition for NBD-derivatives on a Pirkle-type chiral stationary phase, Biomed. Chromatogr. 15 (2001) 227-234. https://doi.org/10.1002/bmc.47
[17] I. Ilisz, R. Berkecz, A. Peter, HPLC separation of amino acid enantiomers and small peptides on macrocyclic antibiotic-based chiral stationary phases: A review, J. Sep. Sci. 29 (2006) 1305-1321. https://doi.org/10.1002/jssc.200600046
[18] M. Lämmerhofer, E. Tobler, E. Zarbl, et al., Macroporous monolithic chiral stationary phases for capillary electrochromatography: New chiral monomer derived from cinchona alkaloid with enhanced enantioselectivity, Electrophoresis 24 (2003) 2986-2999. https://doi.org/10.1002/elps.200305527
[19] L.N. Tran, J.H. Park, Enantiomer separation of acidic chiral compounds on a

quinine-silica/zirconia hybrid monolith by capillary electrochromatography, J. Chromatogr. A 1396 (2015) 140-147. https://doi.org/10.1016/j.chroma.2015.03.085
[20] M. Lämmerhofer, F. Svec, J.M.J. Frechet, Chiral monolithic columns for enantioselective capillary electrochromatography prepared by copolymerization of a monomer with quinidine functionality. 2. Effect of chromatographic conditions on the chiral separations, Anal. Chem. 72 (2000) 4623-4628.
https://doi.org/10.1021/ac000323d
[21] Q.X. Zhang, V. Gil, E. Sánchez-López, et al., Evaluation of the potential of a quinidine-based monolithic column on the enantiomeric separation of herbicides by nano-liquid chromatography, Microchem. J. 123 (2015) 15-21.
https://doi.org/10.1016/j.microc.2015.05.011
[22] H.H. Wu, Q.Q. Wang, M. Ruan, et al., Enantioseparation of N-derivatized amino acids by micro-liquid chromatography/laser induced fluorescence detection using quinidine-based monolithic columns, J. Pharm. Biomed. Anal. 121 (2016) 244-252. https://doi.org/10.1016/j.jpba.2015.12.011
[23] H. Han, Q.Q. Wang, H.H. Wu, et al., Establishment and Application of an Automated Chiral Two-dimensional High Performance Liquid Chromatography Method for Bio-analysis of D-Acidic Amino Acids, Chinese J. Anal. Chem. 42 (2014) 891-895. https://doi.org/10.1016/S1872-2040(14)60746-5
[24] K. Hamase, A. Morikawa, S. Etoh, et al., Analysis of Small Amounts of D-Amino Acids and the Study of Their Physiological Functions in Mammals, Anal. Sci. 25 (2009) 961-968. https://doi.org/10.2116/analsci.25.961
[25] Q.Q. Wang, J. Feng, H. Han, et al., Enantioseparation of N-derivatized amino acids by micro-liquid chromatography using carbamoylated quinidine functionalized monolithic stationary phase, J. Chromatogr. A 1363 (2014) 207-215.
https://doi.org/10.1016/j.chroma.2014.06.039
[26] E. Dominguez-Vega, A.L. Crego, K. Lomsadze, et al., Enantiomeric separation of FMOC-amino acids by nano-LC and CEC using a new chiral stationary phase, cellulose tris(3-chloro-4-methylphenylcarbamate), Electrophoresis 32 (2011) 2700-2707. https://doi.org/10.1002/elps.201000701
[27] L. Chen, Q. Chen, Z.Z. Zhang, et al., A novel colorimetric determination of free amino acids content in tea infusions with 2,4-dinitrofluorobenzene, J. Food Compos. Anal. 22 (2009) 137-141. https://doi.org/10.1016/j.jfca.2008.08.007
[28] F.J. Márquez, A.R. Quesada, F. Sánchez-Jiménez, et al., Determination of 27

dansyl amino acid derivatives in biological fluids by reversed-phase high-performance liquid chromatography, J. Chromatogr. B 380 (1986) 275-283. https://doi.org/10.1016/S0378-4347(00)83656-7
[29] Q.Q. Wang, P.J. Zhu, M. Ruan, et al., Chiral separation of acidic compounds using an O-9-(tert-butylcarbamoyl)quinidine functionalized monolith in micro-liquid chromatography, J. Chromatogr. A 1444 (2016) 64-73. https://doi.org/10.1016/j.chroma.2016.03.047
[30] Q.Q. Wang, E. Sánchez-López, H. Han, et al., Separation of N-derivatized di- and tri-peptide stereoisomers by micro-liquid chromatography using a quinidine-based monolithic column-Analysis of L-carnosine in dietary supplements, J. Chromatogr. A 1428 (2016) 176-184. https://doi.org/10.1016/j.chroma.2015.09.016
[31] J.L. Guo, Y. Xiao, Y.J. Lin, et al., Effect of the crosslinker type on the enantioseparation performance of β-cyclodextrin functionalized monoliths prepared by the one-pot approach, J. Chromatogr. A 1467 (2016) 288-296. https://doi.org/10.1016/j.chroma.2016.05.078
[32] Q.Q. Duan, C.S. Liu, Z.H. Liu, et al., Preparation and evaluation of a novel monolithic column containing double octadecyl chains for reverse-phase micro high performance liquid chromatography, J. Chromatogr. A 1345 (2014) 174-181. https://doi.org/10.1016/j.chroma.2014.04.036
[33] M. Lämmerhofer, F. Svec, J.M.J. Frechet, et al., Monolithic stationary phases for enantioselective capillary electrochromatography, J. Micro. Sep. 12 (2000) 597-602. https://doi.org/10.1002/1520-667X(2000)12:12<597: AID-MCS1005>3.0.CO;2-6
[34] H. Lin, J.J. Ou, Z.B. Zhang, et al., Facile preparation of zwitterionic organic-silica hybrid monolithic capillary column with an improved “one-pot” approach for hydrophilic-interaction liquid chromatography (HILIC), Anal. Chem. 84 (2012) 2721-2728. https://doi.org/10.1021/ac3001429
[35] J.J. Ou, H. Lin, Z.B. Zhang, et al., Recent advances in preparation and application of hybrid organic-silica monolithic capillary columns, Electrophoresis 34 (2013) 126-140. https://doi.org/10.1002/elps.201200344
[36] H.Y. Zhang, J.J. Ou, Z.S. Liu, et al., Preparation of hybrid monolithic columns via “one-pot” photoinitiated thiol-acrylate polymerization for retention-independent performance in capillary liquid chromatography, Anal. Chem. 87 (2015) 8789-8797. https://doi.org/10.1021/acs.analchem.5b01707
[37] M. Kato, H. Saruwatari, K. Sakai-Kato, et al., Silica sol-gel/organic hybrid material

for protein encapsulated column of capillary electrochromatography, J. Chromatogr. A 1044 (2004) 267-270. https://doi.org/10.1016/j.chroma.2004.04.056
[38] D.S. Xu, E. Sánchez-López, Q.Q. Wang, et al., Preparation of an O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine-silica hybrid monolithic column for the enantioseparation of amino acids by nano-liquid chromatography, J. Chromatogr. A 1593 (2019) 63-72. https://doi.org/10.1016/j.chroma.2019.01.065

CHAPTER IV
DISCUSSION

In this PhD Thesis, three different chiral monolithic columns have been developed and one of them has been applied to the determination of L-amino acids in dietary supplements. In this works, the chiral monomers used for polymerization were synthesized, the preparation process for the monolithic columns was optimized, and the characterization of the optimal columns was also carried out. Finally, all of them were evaluated for the enantiomeric separation of a variety of chiral compounds of interest under different chromatographic conditions by nano-LC.

I. Synthesis of the functional monomers

It is well known that the nucleophilic addition of amine or hydroxyl groups was often used for the derivatization of the functional monomers. In this PhD Thesis, since vancomycin and teicoplanin have amino and carboxyl groups on the monomers, we tried the "one-pot" and post-modification at the beginning of our design. However, the monomers could not be bonded to the stationary phase due to the steric space and low reaction efficiency. Hence, the single-step method was chosen to prepare the monolithic columns, and the monomers were firstly modified using ICNEML to react with their primary amino site to bring the ethyl methacrylate group. In brief, vancomycin or teicoplanin were dissolved in DMSO. Then, pyridine and ICNEML were added into the mixture and stirred for 24 h under nitrogen at room temperature. Acetone was added to collect the precipitate which was washed with acetone several times. Finally, the molecular formula of the precipitate was established by HR-ESI-MS after it was dried under vacuum. Regarding the functional monomer MQD, it was also re-synthesized according to a previously reported method, and the purified product was determined by MS spectrum (ESI+).

II. Preparation of the monolithic columns

For the poly(ICNEML-vancomycin-*co*-EDMA) polymer-based monolithic column, the porogen selection is a critical step since the type and amount of porogens influence the porosity, morphology, permeability and the chromatographic efficiency of the monolith. In our initial experiments, a series of commonly used polar and non-polar solvents was investigated in order to solve the problem of the monomers solubility, and to ensure that the monolithic column had good permeability and mechanical stability. As a result, the binary solvent system consisting of MeOH and DMSO was selected as the porogen, and EDMA was chosen as the cross-linker. Later on, polymerization

conditions were systematically optimized, including the study of the influence of the porogens and the content of the monomer mixture.

For the teicoplanin and MQD organic-silica hybrid monolithic columns, to solve monomer solubility and select suitable porogens also remained as the top priority. As MQD can be easily dissolved in MeOH, a series of commonly used polar and non-polar solvents was studied to be combined according to their solubility. Finally, EG was selected for the MQD monolithic column, and the influence of solvent combination, the monomers, the ratio of silanization reagents, and the reaction time and temperature was investigated under the microscope. Due to ICNEML-teicoplanin was easily solved in DMSO and H_2O, the aqueous system containing PEG, urea and HAc was selected to prepare the organic-silica hybrid monolithic column. Moreover, the influence of solvent combination, the monomers, the ratio of silanization reagents, and the reaction time and temperature, were also investigated under the microscope.

III. Characterization and evaluation of the optimal monolithic columns

The optimal monolithic columns developed in this PhD Thesis were characterized and evaluated using SEM, microscopic morphology, permeability, reproducibility, mechanical stability and Van Deemter plots. Results obtained from SEM and microscopic morphology showed that all the resulting monoliths exhibited a uniform dark structure, while the polymer-based monolithic column and organic-silica hybrid monolithic columns showed different morphologies in the SEM results, which was consistent with the results reported in the literature.

In order to investigate the permeability, all the optimal monolithic columns were tested under different solvents, such as 100% MeOH, 100% ACN, 50% ACN aqueous solution and 100% H_2O. Results obtained in this PhD Thesis demonstrated that all columns (organic-silica hybrid monolithic columns and polymer-based monolithic column) showed good permeability. In addition, the swell or shrink in solvents with different polarities was low.

For all the monolithic columns, the reproducibility was evaluated by calculating the RSD values for k_1, k_2, α and Rs on column-to-column, batch-to-batch, run-to-run and day-to-day experiments. As the results showed, all the RSD values did not exceed a 6%, which means that they exhibited good reproducibility.

The mechanical stability and Van Deemter plots were also investigated in the different polar and non-polar solvents. Mechanical stability was evaluated by

calculating the R^2 values obtained for the plots between the linear velocity and the backpressure, and Van Deemter plots for the monolithic columns were obtained by calculating the theoretical plate height for the different linear velocity values.

IV. Optimization of the chromatographic conditions

Another important factor which can affect the enantioseparation is the mobile phase employed. In order to obtain good enantioresolutions, the mobile phase was systematically optimized including polar organic and reversed phase modes. For the POM, the mobile phase was composed of ACN-MeOH and TEA-HAc, and it affected the enantioselectivity by changing the charge-charge interactions, hydrogen bonding and π-π interactions, among other factors. In this PhD Thesis, the MeOH/ACN content, the TEA/HAc ratio and their total concentrations, were systematically optimized to achieve satisfactory enantioresolution, column efficiency and suitable separation time. For the RPM, the influence of the type and the content of organic solvent was investigated. As the results showed, the type and content of the organic solvent had a great effect on the enantioselectivity, column efficiency and separation time. Furthermore, the pH value and concentration of the buffer were also optimized to maximize column efficiency, enantioresolution and separation time.

V. Application of the monolithic columns

All the monolithic columns developed in this PhD Thesis were applied to the enantioseparation of racemic standards or to the analysis of real samples. For the poly(ICNEML-vancomycin-*co*-EDMA) monolithic column, baseline or partial enantioseparation were obtained for series of drugs including thalidomide, colchicine, carteolol, salbutamol, clenbuterol and several other β-blockers under either POM or RPM. Surprisingly (because teicoplanin is a chiral selector similar to vancomycin), the ICNEML-teicoplanin organic-silica hybrid monolithic column was successfully applied to achieve the enantioseparation of amino alcohols, *N*-derivatized amino acids and mandelic acids. 15 out of 20 amino alcohols were baseline separated with good column efficiency in the POM mode, and 3 out of 5 mandelic acids and 5 out of 6 *N*-derivatized amino acids were baseline separated in the RPM mode. For the MQD organic-silica hybrid monolithic column, it was not only applied to enantioseparation of a lot of *N*-derivatized protein or non-protein amino acids, but also to the quantitation of L-tryptophan and L-norvaline in dietary supplements.

CHAPTER V
CONCLUSIONS

From the results obtained in this PhD Thesis, the following general conclusions can be derived:

- Vancomycin and teicoplanin monomers were modified using ICNEML to react with their primary amino sites to bring the ethyl methacrylate group.

- A facile single-step preparation strategy was developed for fabricating vancomycin functionalized organic polymer-based monolith within 100 μm fused-silica capillary. The synthetic chiral functional monomer, i.e 2-isocyanatoethyl methacrylate (ICNEML) derivative of vancomycin, was co-polymerized with the cross-linker ethylene dimethacrylate (EDMA) in the presence of methanol and dimethyl sulfoxide as the selected porogens.

- A novel Tei-ICNEML functionalized organic-silica hybrid monolithic column was successfully developed through a "single-step" strategy by mixing the copolymerization monomer teicoplanin-2-isocyanatoethyl methacrylate (Tei-ICNEML) and initiator into the hydrolysis solution of TMOS and γ-MAPS.

- A straightforward "one-step" strategy was used to prepare a MQD organic silica hybrid monolithic column within a 100 μm I.D. capillary. The polymerization solvent was prepared by mixing MQD, MeOH, EG, CTAB, H_2O, $NH_3 \cdot H_2O$, TMOS, VTMS, and AIBN through just one step.

- Adequate reproducibility, mechanical stability, permeability, and column morphology were observed for the optimized poly(ICNEML-vancomycin-*co*-EDMA) monolithic column, Tei-ICNEML and MQD organic-silica hybrid monolithic columns.

- The influence of the mobile phase composition (buffer pH, organic modifier content and buffer concentration) on the enantioseparation was further investigated both in the polar organic and reversed phase modes.

- The vancomycin functionalized polymer monolithic column poly(ICNEML-vancomycin-*co*-EDMA) displayed baseline or partial enantioseparation of

series of drugs including thalidomide, colchicine, carteolol, salbutamol, clenbuterol and several other β-blockers using MeOH/ACN/TEA/HAc (85:15:0.08:0.02, v/v/v/v), 0.5% TEAA (pH=5.5)/MeOH (10/90, v/v), 50 mM ammonium acetate (pH=5.5)/water/ACN (5/5/90, v/v/v) and 0.5% TEAA buffer (pH=5.4)/ACN (70/30, v/v) mobile phase conditions. The proposed single-step approach not only resulted in a vancomycin functionalized organic polymer-based monolithic column with acceptable performance, but also significantly simplified the preparation procedure by reducing time and labor.

- The Tei-ICNEML functionalized organic-silica monolithic column was successfully applied to achieve the enantioseparation of amino alcohols, *N*-derivatized amino acids and mandelic acids. 15 out of 20 amino alcohols were baseline separated with good column efficiency using a MeOH/ACN/TEA/HAc (80:20:0.03:0.03, v/v/v/v) mobile phase, and 3 out of 5 mandelic acids, and 5 out of 6 *N*-derivatized amino acids, were baseline separated using a MeOH/ACN/TEA/HAc (80:20:0.03:0.03, v/v/v/v) mobile phase. Compared with the polymer based teicoplanin functionalized monolithic column poly(ICNEML-Teicoplanin-*co*-EDMA), the Tei-ICNEML organic-silica hybrid monolithic column exhibited higher column efficiency and enantioresolution using MeOH/ACN/TEA/HAc (80:20:0.03:0.03, v/v/v/v) (POM) and MeOH/ACN/TEA/HAc (80:20:0.03:0.03, v/v/v/v) (RPM) mobile phases except for mandelic acids. Moreover, they had almost the same selectivity for the amino alcohols in POM, while Tei-ICNEML organic-silica hybrid monolithic column exhibited higher selectivity for the N- derivatized amino acids in RPM.

- The MQD functionalized organic-silica monolithic column displayed excellent column efficiency, enantioselectivity and enantioresolution for 52 N-derivatized protein and non-protein amino acids, and 44 out of them were baseline separated under the optimized conditions in either 10 mM ammonium acetate/ACN (30/70, v/v) (apparent pH=5.3) (RPM) or ACN/MeOH/HAc/TEA (60/40/0.055/0.005, v/v/v/v) (POM), and they exhibited similar α values in both RPM and POM.

- Comparing the α values for all the amino acids in the same mode, the higher electrophilic character of the N-protecting groups would lead to a higher

enantioselectivity, following this trend: 3,5-DNB-leucine (α = 4.22)> 3,5-DCIB-leucine (α = 2.62)> 3,5-DMB-leucine (α = 1.91)> *m*-CIB-leucine (α = 1.80)> *p*-NB-leucine (α = 1.73)> *p*-CIB-leucine (α=1.58)> B-leucine (α=1.33). However, the enantioresolution and column efficiency values were higher when using ACN/MeOH/HAc/TEA (60/40/0.055/0.005, v/v/v/v) than with 10 mM ammonium acetate/ACN (30/70, v/v) (apparent pH=5.3).

- Most of FMOC-derivatized amino acids were not baseline enantioseparated in any of the ACN/MeOH/HAc/TEA (60/40/0.055/0.005, v/v/v/v) (POM) and 10 mM ammonium acetate/ACN (30/70, v/v) (apparent pH=5.3) (RPM) mobile phase conditions while analysis times were shorter in POM when compared to RPM for all the N-derivatized amino acids tested.

- 27 FMOC derivatized amino acids including 19 protein and 8 non-protein amino acids were re-tested using a 3 mM ammonium acetate (apparent pH=4.8)/ACN (35/65, v/v) mobile phase, and 19 out of them were enantiomerically discriminated obtaining baseline separation for 11 of them.

- The MQD organic-silica hybrid monolithic column was applied to the development of an analytical methodology enabling the determination of L-trytophan and L-norvaline in dietary supplements. The method was proven to be adequate in terms of selectivity, linearity, precision, accuracy and LODs and LOQs for the enantiomeric determination of L-norvaline and L-tryptophan in dietary supplements. The developed strategy can be used for the quality control of dietary supplements in a fast and accurate manner.

CHAPTER VI
BIBLIOGRAPHY

References for the Introduction:

[1] F. Svec, J.M. Fréchet, Continuous rods of macroporous polymer as high-performance liquid chromatography separation media, Analytical Chemistry 64 (1992) 820-822.

[2] D. Mould, R. Synge, Electrokinetic ultrafiltration analysis of polysaccharides. A new approach to the chromatography of large molecules, Analyst 77 (1952) 964-969.

[3] B. Chankvetadze, Monolithic chiral stationary phases for liquid-phase enantioseparation techniques, Journal of separation science 33 (2010) 305-314.

[4] Z. Jiang, N.W. Smith, P.D. Ferguson, M.R. Taylor, Novel highly hydrophilic zwitterionic monolithic column for hydrophilic interaction chromatography, Journal of separation science 32 (2009) 2544-2555.

[5] Q. Wang, H. Wu, K. Peng, H. Jin, H. Shao, Y. Wang, J. Crommen, Z. Jiang, Hydrophilic polymeric monoliths containing choline phosphate for separation science applications, Analytica chimica acta 999 (2018) 184-189.

[6] A. Ghanem, M. Ahmed, H. Ishii, T. Ikegami, Immobilized β-cyclodextrin-based silica vs polymer monoliths for chiral nano liquid chromatographic separation of racemates, Talanta 132 (2015) 301-314.

[7] J.-S. Hong, J.H. Park, Chiral separation of basic compounds on sulfated β-cyclodextrin-coated zirconia monolith by capillary electrochromatography, Bulletin of the Korean Chemical Society 34 (2013) 1809-1813.

[8] Z. Zhang, M. Wu, R.a. Wu, J. Dong, J. Ou, H. Zou, Preparation of perphenylcarbamoylated β-cyclodextrin-silica hybrid monolithic column with "one-pot" approach for enantioseparation by capillary liquid chromatography, Analytical chemistry 83 (2011) 3616-3622.

[9] Y. Li, C. Song, L. Zhang, W. Zhang, H. Fu, Fabrication and evaluation of chiral monolithic column modified by β-cyclodextrin derivatives, Talanta 80 (2010) 1378-1384.

[10] Z. Liu, Y. Peng, T. Wang, G. Yuan, Q. Zhang, J. Guo, Z. Jiang, Preparation and application of novel zwitterionic monolithic column for hydrophilic interaction chromatography, Journal of separation science 36 (2013) 262-269.

[11] M. Lämmerhofer, E.C. Peters, C. Yu, F. Svec, J.M. Fréchet, W. Lindner, Chiral monolithic columns for enantioselective capillary electrochromatography prepared by

copolymerization of a monomer with quinidine functionality. 1. Optimization of polymerization conditions, porous properties, and chemistry of the stationary phase, Analytical chemistry 72 (2000) 4614-4622.

[12] K. Nakanishi, H. Minakuchi, N. Soga, N. Tanaka, Structure design of double-pore silica and its application to HPLC, Journal of sol-gel science and technology 13 (1998) 163-169.

[13] J. Rozenbrand, W.P. van Bennekom, Silica-based and organic monolithic capillary columns for LC: Recent trends in proteomics, Journal of separation science 34 (2011) 1934-1944.

[14] R.D. Arrua, M. Talebi, T.J. Causon, E.F. Hilder, Review of recent advances in the preparation of organic polymer monoliths for liquid chromatography of large molecules, Analytica Chimica Acta 738 (2012) 1-12.

[15] J.D. Hayes, A. Malik, Sol− gel monolithic columns with reversed electroosmotic flow for capillary electrochromatography, Analytical chemistry 72 (2000) 4090-4099.

[16] H. Zhang, J. Ou, Z. Liu, H. Wang, Y. Wei, H. Zou, Preparation of hybrid monolithic columns via “one-pot” photoinitiated thiol–acrylate polymerization for retention-independent performance in capillary liquid chromatography, Analytical chemistry 87 (2015) 8789-8797.

[17] Z. Zhang, H. Lin, J. Ou, H. Qin, R.a. Wu, J. Dong, H. Zou, Preparation of phenyl-silica hybrid monolithic column with “one-pot” process for capillary liquid chromatography, Journal of Chromatography A 1228 (2012) 263-269.

[18] R. Yuan, Y. Wang, G. Ding, Enantiomeric separation by capillary electrochromatography on a sulfated poly β-cyclodextrin modified silica-based monolith, Analytical Sciences 26 (2010) 943-947.

[19] T. Koide, K. Ueno, Enantiomeric separations by capillary electrochromatography with charged polyacrylamide gels incorporating chiral selectors, Analytical sciences 16 (2000) 1065-1070.

[20] M. Pumera, I. Jelinek, J. Jindrich, O. Benada, β-Cyclodextrin-modified monolithic stationary phases for capillary electrochromatography and nano-HPLC chiral analysis of ephedrine and ibuprofen, Journal of liquid chromatography & related technologies 25 (2002) 2473-2484.

[21] D. Wistuba, V. Schurig, Enantiomer separation by capillary

electrochromatography on a cyclodextrin-modified monolith, ELECTROPHORESIS: An International Journal 21 (2000) 3152-3159.

[22] D. Wistuba, L. Banspach, V. Schurig, Enantiomeric separation by capillary electrochromatography using monolithic capillaries with sol-gel-glued cyclodextrin-modified silica particles, Electrophoresis 26 (2005) 2019-2026.

[23] K. Cabrera, Applications of silica-based monolithic HPLC columns, Journal of separation science 27 (2004) 843-852.

[24] K. Nakanishi, N. Soga, Phase separation in gelling silica–organic polymer solution: systems containing poly (sodium styrenesulfonate), Journal of the American Ceramic Society 74 (1991) 2518-2530.

[25] H. Minakuchi, K. Nakanishi, N. Soga, N. Ishizuka, N. Tanaka, Octadecylsilylated porous silica rods as separation media for reversed-phase liquid chromatography, Analytical chemistry 68 (1996) 3498-3501.

[26] S.M. Fields, Silica xerogel as a continuous column support for high-performance liquid chromatography, Analytical chemistry 68 (1996) 2709-2712.

[27] N. Ishizuka, H. Minakuchi, K. Nakanishi, N. Soga, K. Hosoya, N. Tanaka, Chromatographic properties of miniaturized silica rod columns, Journal of High Resolution Chromatography 21 (1998) 477-479.

[28] M.T. Dulay, R.P. Kulkarni, R.N. Zare, Preparation and characterization of monolithic porous capillary columns loaded with chromatographic particles, Analytical chemistry 70 (1998) 5103-5107.

[29] R.a. Wu, L. Hu, F. Wang, M. Ye, H. Zou, Recent development of monolithic stationary phases with emphasis on microscale chromatographic separation, Journal of Chromatography A 1184 (2008) 369-392.

[30] A. Ghanem, T. Ikegami, Recent advances in silica-based monoliths: preparations, characterizations and applications, Journal of separation science 34 (2011) 1945.

[31] F. Svec, Quest for organic polymer-based monolithic columns affording enhanced efficiency in high performance liquid chromatography separations of small molecules in isocratic mode, Journal of Chromatography A 1228 (2012) 250-262.

[32] P. Jandera, Advances in the development of organic polymer monolithic columns and their applications in food analysis—a review, Journal of chromatography A 1313 (2013) 37-53.

[33] J. Ou, Z. Liu, H. Wang, H. Lin, J. Dong, H. Zou, Recent development of hybrid organic-silica monolithic columns in CEC and capillary LC, Electrophoresis 36 (2015) 62-75.

[34] T. Zhu, K.H. Row, Preparation and applications of hybrid organic–inorganic monoliths: a review, Journal of separation science 35 (2012) 1294-1302.

[35] J. Ou, H. Lin, Z. Zhang, G. Huang, J. Dong, H. Zou, Recent advances in preparation and application of hybrid organic-silica monolithic capillary columns, Electrophoresis 34 (2013) 126-140.

[36] J. Guo, Y. Lin, Y. Xiao, J. Crommen, Z. Jiang, Recent developments in cyclodextrin functionalized monolithic columns for the enantioseparation of chiral drugs, Journal of pharmaceutical and biomedical analysis 130 (2016) 110-125.

[37] D. Wistuba, V. Schurig, Recent progress in enantiomer separation by capillary electrochromatography, ELECTROPHORESIS: An International Journal 21 (2000) 4136-4158.

[38] J.-M. Park, J.H. Park, Enantiomer separations of basic chiral compounds by capillary electrochromatography on a phosphated β-cyclodextrin-modified zirconia monolith, Journal of Chromatography A 1339 (2014) 229-233.

[39] T. Koide, K. Ueno, Enantiomeric separations of cationic and neutral compounds by capillary electrochromatography with charged polyacrylamide gels incorporating chiral selectors, Analytical sciences 14 (1998) 1021-1023.

[40] M. Guerrouache, M.C. Millot, B. Carbonnier, Functionalization of macroporous organic polymer monolith based on succinimide ester reactivity for chiral capillary chromatography: a cyclodextrin click approach, Macromolecular rapid communications 30 (2009) 109-113.

[41] Y. Lv, D. Mei, X. Pan, T. Tan, Preparation of novel β-cyclodextrin functionalized monolith and its application in chiral separation, Journal of Chromatography B 878 (2010) 2461-2464.

[42] J. Guo, Q. Zhang, Y. Peng, Z. Liu, L. Rao, T. He, J. Crommen, P. Sun, Z. Jiang, A facile and efficient one-step strategy for the preparation of β-cyclodextrin monoliths, Journal of separation science 36 (2013) 2441-2449.

[43] A. Salwiński, V. Roy, L.A. Agrofoglio, R. Delépée, In situ one-step method for synthesis of “click”-functionalized monolithic stationary phase for capillary

electrochromatography, Macromolecular Chemistry and Physics 212 (2011) 2700-2707.

[44] Q. Zhang, J. Guo, F. Wang, J. Crommen, Z. Jiang, Preparation of a β-cyclodextrin functionalized monolith via a novel and simple one-pot approach and application to enantioseparations, Journal of Chromatography A 1325 (2014) 147-154.

[45] Y. Tian, C. Zhong, E. Fu, Z. Zeng, Novel β-cyclodextrin derivative functionalized polymethacrylate-based monolithic columns for enantioselective separation of ibuprofen and naproxen enantiomers in capillary electrochromatography, Journal of Chromatography A 1216 (2009) 1000-1007.

[46] M. Ahmed, A. Ghanem, Chiral β-cyclodextrin functionalized polymer monolith for the direct enantioselective reversed phase nano liquid chromatographic separation of racemic pharmaceuticals, Journal of Chromatography A 1345 (2014) 115-127.

[47] F.G. Adly, N.Y. Antwi, A. Ghanem, Cyclodextrin-functionalized monolithic capillary columns: preparation and chiral applications, Chirality 28 (2016) 97-109.

[48] J. Guo, Q. Zhang, Z. Yao, X. Zhao, D. Ran, J. Crommen, Z. Jiang, One-step strategy for the synthesis of a derivatized cyclodextrin-based monolithic column, Journal of separation science 37 (2014) 1720-1727.

[49] T. Koide, K. Ueno, Enantiomeric separations of cationic and neutral compounds by capillary electrochromatography with monolithic chiral stationary phases of β-cyclodextrin-bonded negatively charged polyacrylamide gels, Journal of Chromatography A 893 (2000) 177-187.

[50] T. Koide, K. Ueno, Enantiomeric Separations of Acidic and Neutral Compounds by Capillary Electrochromatography with β-Cyclodextrin-Bonded Positively Charged Polyacrylamide Gels, Journal of High Resolution Chromatography 23 (2000) 59-66.

[51] T. Koide, K. Ueno, Enantiomeric separations of cationic and neutral compounds by capillary electrochromatography with β-cyclodextrin-bonded charged polyacrylamide gels, Analytical sciences 15 (1999) 791-794.

[52] M. Li, M. Tarawally, X. Liu, X. Liu, L. Guo, L. Yang, G. Wang, Application of cyclodextrin-modified gold nanoparticles in enantioselective monolith capillary electrochromatography, Talanta 109 (2013) 1-6.

[53] Z. Chen, H. Ozawa, K. Uchiyama, T. Hobo, Cyclodextrin-modified monolithic columns for resolving dansyl amino acid enantiomers and positional isomers by capillary electrochromatography, Electrophoresis 24 (2003) 2550-2558.

[54] S. El Deeb, P. Hasemann, H. Wätzig, Strategies in method development to quantify enantiomeric impurities using CE, Electrophoresis 29 (2008) 3552-3562.

[55] Y. Okamoto, T. Ikai, J. Shen, Controlled immobilization of polysaccharide derivatives for efficient chiral separation, Israel Journal of Chemistry 51 (2011) 1096-1106.

[56] B. Chankvetadze, T. Kubota, T. Ikai, C. Yamamoto, M. Kamigaito, N. Tanaka, K. Nakanishi, Y. Okamoto, High-performance liquid chromatographic enantioseparations on capillary columns containing crosslinked polysaccharide phenylcarbamate derivatives attached to monolithic silica, Journal of separation science 29 (2006) 1988-1995.

[57] F. Qin, C. Xie, S. Feng, J. Ou, L. Kong, M. Ye, H. Zou, Monolithic silica capillary column with coated cellulose tris (3, 5-dimethylphenylcarbamate) for capillary electrochromatographic separation of enantiomers, Electrophoresis 27 (2006) 1050-1059.

[58] B. Chankvetadze, C. Yamamoto, M. Kamigaito, N. Tanaka, K. Nakanishi, Y. Okamoto, High-performance liquid chromatographic enantioseparations on capillary columns containing monolithic silica modified with amylose tris (3, 5-dimethylphenylcarbamate), Journal of Chromatography A 1110 (2006) 46-52.

[59] B. Chankvetadze, T. Ikai, C. Yamamoto, Y. Okamoto, High-performance liquid chromatographic enantioseparations on monolithic silica columns containing a covalently attached 3, 5-dimethylphenylcarbamate derivative of cellulose, Journal of Chromatography A 1042 (2004) 55-60.

[60] T. Ikai, C. Yamamoto, M. Kamigaito, Y. Okamoto, Immobilized-type chiral packing materials for HPLC based on polysaccharide derivatives, Journal of Chromatography B 875 (2008) 2-11.

[61] B. Chankvetadze, C. Yamamoto, Y. Okamoto, Very fast enantioseparation in high-performance liquid chromatography using cellulose tris (3, 5-dimethylphenylcarbamate) coated on monolithic silica support, Chemistry letters 32 (2003) 850-851.

[62] C. Fanali, S. Fanali, B. Chankvetadze, HPLC separation of enantiomers of some flavanone derivatives using polysaccharide-based chiral selectors covalently immobilized on silica, Chromatographia 79 (2016) 119-124.

[63] X. Dong, R.a. Wu, J. Dong, M. Wu, Y. Zhu, H. Zou, The covalently bonded

cellulose tris (3, 5-dimethylphenylcarbamate) on a silica monolithic capillary column for enantioseparation in capillary electrochromatography, Journal of Chromatography B 875 (2008) 317-322.

[64] X. Dong, R. Wu, J. Dong, M. Wu, Y. Zhu, H. Zou, Polyacrylamide-based monolithic capillary column with coating of cellulose tris (3, 5-dimethylphenyl-carbamate) for enantiomer separation in capillary electrochromatography, Electrophoresis 29 (2008) 919-927.

[65] J. Ou, H. Lin, S. Tang, Z. Zhang, J. Dong, H. Zou, Hybrid monolithic columns coated with cellulose tris (3, 5-dimethylphenyl-carbamate) for enantioseparations in capillary electrochromatography and capillary liquid chromatography, Journal of Chromatography A 1269 (2012) 372-378.

[66] A.P. Kumar, J.H. Park, Enantioseparation on cellulose dimethylphenylcarbamate-modified zirconia monolithic columns by reversed-phase capillary electrochromatography, Journal of Chromatography A 1217 (2010) 4494-4500.

[67] A.P. Kumar, J.H. Park, Fast separations of chiral β-blockers on a cellulose tris (3, 5-dimethylphenylcarbamate)-coated zirconia monolithic column by capillary electrochromatography, Journal of Chromatography A 1218 (2011) 5369-5373.

[68] A.P. Kumar, J.H. Park, Chiral separation of basic compounds on a cellulose 3, 5-dimethylphenylcarbamate-coated zirconia monolithin basic eluents by capillary electrochromatography, Journal of Chromatography A 1218 (2011) 6548-6553.

[69] Y. Liu, Y.V. Heyden, D. Mangelings, Amylose-3, 5-dimethylphenylcarbamate immobilized on monolithic silica stationary phases for chiral separations in capillary electrochromatography, Electrophoresis 33 (2012) 1613-1623.

[70] A. Fouad, A.A. Marzouk, S.M. Ibrahim, M. Sobhy, A. Ghanem, Functionalized polymer monoliths with carbamylated amylose for the enantioselective reversed phase nano-liquid chromatographic separation of a set of racemic pharmaceuticals, Journal of Chromatography A 1515 (2017) 91-99.

[71] H. Lin, J. Ou, S. Tang, Z. Zhang, J. Dong, Z. Liu, H. Zou, Facile preparation of a stable and functionalizable hybrid monolith via ring-opening polymerization for capillary liquid chromatography, Journal of Chromatography A 1301 (2013) 131-138.

[72] Y. Zheng, X. Wang, Y. Ji, Monoliths with proteins as chiral selectors for enantiomer separation, Talanta 91 (2012) 7-17.

[73] C. Bi, X. Zheng, S. Azaria, S. Beeram, Z. Li, D. Hage, Chromatographic studies of protein-based chiral separations, Separations 3 (2016) 27.

[74] R. Mallik, T. Jiang, D.S. Hage, High-performance affinity monolith chromatography: development and evaluation of human serum albumin columns, Analytical chemistry 76 (2004) 7013-7022.

[75] R. Mallik, D.S. Hage, Development of an affinity silica monolith containing human serum albumin for chiral separations, Journal of pharmaceutical and biomedical analysis 46 (2008) 820-830.

[76] C. Yao, L. Qi, J. Qiao, H. Zhang, F. Wang, Y. Chen, G. Yang, High-performance affinity monolith chromatography for chiral separation and determination of enzyme kinetic constants, Talanta 82 (2010) 1332-1337.

[77] T. Hong, Y. Zheng, W. Hu, Y. Ji, Preparation and evaluation of bovine serum albumin immobilized chiral monolithic column for affinity capillary electrochromatography, Analytical biochemistry 464 (2014) 43-50.

[78] M. Kato, N. Matsumoto, K. Sakai-Kato, T. Toyo'oka, Investigation of chromatographic performances and binding characteristics of BSA-encapsulated capillary column prepared by the sol–gel method, Journal of pharmaceutical and biomedical analysis 30 (2003) 1845-1850.

[79] K. Sakai-Kato, M. Kato, H. Nakakuki, T. Toyo'oka, Investigation of structure and enantioselectivity of BSA-encapsulated sol–gel columns prepared for capillary electrochromatography, Journal of pharmaceutical and biomedical analysis 31 (2003) 299-309.

[80] M. Kato, K. Sakai-Kato, N. Matsumoto, T. Toyo'oka, A protein-encapsulation technique by the sol− gel method for the preparation of monolithic columns for capillary electrochromatography, Analytical chemistry 74 (2002) 1915-1921.

[81] R. Mallik, H. Xuan, D.S. Hage, Development of an affinity silica monolith containing α1-acid glycoprotein for chiral separations, Journal of Chromatography A 1149 (2007) 294-304.

[82] M. Ahmed, A. Ghanem, Enantioselective Nano Liquid Chromatographic Separation of Racemic Pharmaceuticals: A Facile One-Pot In Situ Preparation of Lipase-Based Polymer Monoliths in Capillary Format, Chirality 26 (2014) 754-763.

[83] T. Hong, X. Chen, Y. Xu, X. Cui, R. Bai, C. Jin, R. Li, Y. Ji, Preparation of

graphene oxide-modified affinity capillary monoliths based on three types of amino donor for chiral separation and proteolysis, Journal of Chromatography A 1456 (2016) 249-256.

[84] R. Gotti, J. Fiori, E. Calleri, C. Temporini, D. Lubda, G. Massolini, Chiral capillary liquid chromatography based on penicillin G acylase immobilized on monolithic epoxy silica column, Journal of Chromatography A 1234 (2012) 45-49.

[85] L. Zhao, L. Yang, Q. Wang, Silica-based polypeptide-monolithic stationary phase for hydrophilic chromatography and chiral separation, Journal of Chromatography A 1446 (2016) 125-133.

[86] J. Sproß, A. Sinz, Monolithic columns with immobilized monomeric avidin: preparation and application for affinity chromatography, Analytical and bioanalytical chemistry 402 (2012) 2395-2405.

[87] S. Xu, Y. Wang, Y. Tang, Y. Ji, A protein-based mixed selector chiral monolithic stationary phase in capillary electrochromatography, New Journal of Chemistry 42 (2018) 13520-13528.

[88] G. Massolini, C. Temporini, E. Calleri, Penicillin G acylase as chiral selector in LC and CE: Exploring the origins of enantioselectivity, Journal of Chromatography B 875 (2008) 20-29.

[89] C. Miao, R. Bai, S. Xu, T. Hong, Y. Ji, Carboxylated single-walled carbon nanotube-functionalized chiral polymer monoliths for affinity capillary electrochromatography, Journal of Chromatography A 1487 (2017) 227-234.

[90] S. Xu, R. Mo, C. Jin, X. Cui, R. Bai, Y. Ji, Mesoporous silica nanoparticles incorporated hybrid monolithic stationary phase immobilized with pepsin for enantioseparation by capillary electrochromatography, Journal of pharmaceutical and biomedical analysis 140 (2017) 190-198.

[91] D.W. Armstrong, Y. Tang, S. Chen, Y. Zhou, C. Bagwill, J.-R. Chen, Macrocyclic antibiotics as a new class of chiral selectors for liquid chromatography, Analytical Chemistry 66 (1994) 1473-1484.

[92] M.G. Schmid, J. Koidl, C. Freigassner, S. Tahedl, L. Wojcik, T. Beesley, D.W. Armstrong, G. Gübitz, New particle-loaded monoliths for chiral capillary electrochromatographic separation, Electrophoresis 25 (2004) 3195-3203.

[93] O. Kornyšova, P.K. Owens, A. Maruška, Continuous beds with vancomycin as

chiral stationary phase for capillary electrochromatography, Electrophoresis 22 (2001) 3335-3338.

[94] O. Kornyšova, R. Jarmalavičienė, A. Maruška, A simplified synthesis of polymeric nonparticulate stationary phases with macrocyclic antibiotic as chiral selector for capillary electrochromatography, Electrophoresis 25 (2004) 2825-2829.

[95] X. Dong, J. Dong, J. Ou, Y. Zhu, H. Zou, Preparation and evaluation of a vancomycin-immobilized silica monolith as chiral stationary phase for CEC, Electrophoresis 28 (2007) 2606-2612.

[96] M.-L. Hsieh, L.-K. Chau, Y.-S. Hon, Single-step approach for fabrication of vancomycin-bonded silica monolith as chiral stationary phase, Journal of Chromatography A 1358 (2014) 208-216.

[97] E. Pittler, M.G. Schmid, Enantioseparation of dansyl amino acids by HPLC on a monolithic column dynamically coated with a vancomycin derivative, Biomedical Chromatography 24 (2010) 1213-1219.

[98] G.-S. Ding, A.-N. Tang, Capillary electrochromatographic separation of racemates on a norvancomycin-bonded monolithic column under reversed-phase conditions, Journal of Chromatography A 1208 (2008) 232-238.

[99] A.N. Tang, X.N. Wang, G.S. Ding, X.P. Yan, On-line preconcentration and enantioseparation of thalidomide racemates by CEC with the hyphenation of octyl and norvancomycin monoliths, Electrophoresis 30 (2009) 682-688.

[100] M. Kim, J.H. Park, Enantioseparation of chiral acids and bases on a clindamycin phosphate-modified zirconia monolith by capillary electrochromatography, Journal of Chromatography A 1251 (2012) 244-248.

[101] L.N. Tran, S. Dixit, J.H. Park, Enantioseparation of basic chiral compounds on a clindamycin phosphate-silica/zirconia hybrid monolith by capillary electrochromatography, Journal of Chromatography A 1356 (2014) 289-293.

[102] S. Dixit, J.H. Park, Enantioseparation of basic chiral drugs on a carbamoylated erythromycin-zirconia hybrid monolith using capillary electrochromatography, Journal of Chromatography A 1416 (2015) 129-136.

[103] S. Dixit, I.S. Lee, J.H. Park, Carbamoylated azithromycin incorporated zirconia hybrid monolith for enantioseparation of acidic chiral drugs using non-aqueous capillary electrochromatography, Journal of Chromatography A 1507 (2017) 132-140.

[104] M.G. Schmid, G. Gübitz, Enantioseparation by chromatographic and electromigration techniques using ligand-exchange as chiral separation principle, Analytical and bioanalytical chemistry 400 (2011) 2305-2316.

[105] M.G. Schmid, J. Koidl, P. Wank, G. Kargl, H. Zöhrer, G. Gübitz, Enantioseparation by ligand-exchange using particle-loaded monoliths: capillary-LC versus capillary electrochromatography, Journal of biochemical and biophysical methods 70 (2007) 77-85.

[106] Z.A. Al-Othman, A. Al-Warthan, I. Ali, Advances in enantiomeric resolution on monolithic chiral stationary phases in liquid chromatography and electrochromatography, Journal of separation science 37 (2014) 1033-1057.

[107] Z. Chen, T. Hobo, Chemically L-prolinamide-modified monolithic silica column for enantiomeric separation of dansyl amino acids and hydroxy acids by capillary electrochromatography and μ-high performance liquid chromatography, Electrophoresis 22 (2001) 3339-3346.

[108] S. Mizrahi, D. Rizkov, A.I. Shames, O. Lev, Chiral separation of dansyl amino acids by ligand exchange capillary electrochromatography in a low molecular weight organogel, Electrophoresis 29 (2008) 3941-3948.

[109] M.G. Schmid, N. Grobuschek, C. Tuscher, G. Gübitz, Á. Végvári, E. Machtejevas, A. Maruška, S. Hjertén, Chiral separation of amino acids by ligand-exchange capillary electrochromatography using continuous beds, ELECTROPHORESIS: An International Journal 21 (2000) 3141-3144.

[110] O. Lecnik, G. Gübitz, M.G. Schmid, Role of the charge in continuous beds in the chiral separation of hydroxy acids by ligand-exchange capillary electrochromatography, Electrophoresis 24 (2003) 2983-2985.

[111] P. Puchalska, E. Pittler, M. Trojanowicz, G. Gübitz, M.G. Schmid, Enantioseparation of amino acids and α-hydroxy acids on ligand-exchange continuous beds by capillary electrochromatography, Electrophoresis 31 (2010) 1517-1520.

[112] M.G. Schmid, N. Grobuschek, O. Lecnik, G. Gübitz, Á. Végvári, S. Hjertén, Enantioseparation of hydroxy acids on easy-to-prepare continuous beds for capillary electrochromatography, Electrophoresis 22 (2001) 2616-2619.

[113] M.G. Schmid, K. Schreiner, D. Reisinger, G. Gübitz, Fast chiral separation by ligand-exchange HPLC using a dynamically coated monolithic column, Journal of

separation science 29 (2006) 1470-1475.

[114] Z. Chen, T. Hobo, Chemically l-phenylalaninamide-modified monolithic silica column prepared by a sol− gel process for enantioseparation of dansyl amino acids by ligand exchange-capillary electrochromatography, Analytical chemistry 73 (2001) 3348-3357.

[115] Z. Chen, M. Niitsuma, K. Uchiyama, T. Hobo, Comparison of enantioseparations using Cu (II) complexes with l-amino acid amides as chiral selectors or chiral stationary phases by capillary electrophoresis, capillary electrochromatography and micro liquid chromatography, Journal of Chromatography A 990 (2003) 75-82.

[116] Z. Chen, K. Uchiyama, T. Hobo, Chemically modified chiral monolithic silica column prepared by a sol–gel process for enantiomeric separation by micro high-performance liquid chromatography, Journal of Chromatography A 942 (2002) 83-91.

[117] Z. Chen, T. Nishiyama, K. Uchiyama, T. Hobo, Electrochromatographic enantioseparation using chiral ligand exchange monolithic sol–gel column, Analytica chimica acta 501 (2004) 17-23.

[118] M. Lämmerhofer, Chiral recognition by enantioselective liquid chromatography: mechanisms and modern chiral stationary phases, Journal of Chromatography A 1217 (2010) 814-856.

[119] M. Wolter, M. Lämmerhofer, In-situ functionalized monolithic polysiloxane-polymethacrylate composite materials from polythiol-ene double click reaction in capillary column format for enantioselective nano-high-performance liquid chromatography, Journal of Chromatography A 1497 (2017) 172-179.

[120] E.J. Carrasco-Correa, G. Ramis-Ramos, J.M. Herrero-Martínez, M. Lämmerhofer, Polymethacrylate monoliths with immobilized poly-3-mercaptopropyl methylsiloxane film for high-coverage surface functionalization by thiol-ene click reaction, Journal of Chromatography A 1367 (2014) 123-130.

[121] M. Lämmerhofer, F. Svec, J.M. Fréchet, W. Lindner, Chiral monolithic columns for enantioselective capillary electrochromatography prepared by copolymerization of a monomer with quinidine functionality. 2. Effect of chromatographic conditions on the chiral separations, Analytical chemistry 72 (2000) 4623-4628.

[122] M. Lämmerhofer, F. Svec, J.M. Fréchet, W. Lindner, Monolithic stationary phases for enantioselective capillary electrochromatography, Journal of Microcolumn

Separations 12 (2000) 597-602.

[123] M. Lämmerhofer, E. Tobler, E. Zarbl, W. Lindner, F. Svec, J.M. Fréchet, Macroporous monolithic chiral stationary phases for capillary electrochromatography: New chiral monomer derived from cinchona alkaloid with enhanced enantioselectivity, Electrophoresis 24 (2003) 2986-2999.

[124] B. Preinerstorfer, W. Bicker, W. Lindner, M. Lämmerhofer, Development of reactive thiol-modified monolithic capillaries and in-column surface functionalization by radical addition of a chromatographic ligand for capillary electrochromatography, Journal of Chromatography A 1044 (2004) 187-199.

[125] M. Lämmerhofer, A. Gargano, Monoliths with chiral surface functionalization for enantioselective capillary electrochromatography, Journal of pharmaceutical and biomedical analysis 53 (2010) 1091-1123.

[126] Q. Wang, J. Feng, H. Han, P. Zhu, H. Wu, M.L. Marina, J. Crommen, Z. Jiang, Enantioseparation of N-derivatized amino acids by micro-liquid chromatography using carbamoylated quinidine functionalized monolithic stationary phase, Journal of Chromatography A 1363 (2014) 207-215.

[127] Q. Wang, E. Sánchez-López, H. Han, H. Wu, P. Zhu, J. Crommen, M.L. Marina, Z. Jiang, Separation of N-derivatized di-and tri-peptide stereoisomers by micro-liquid chromatography using a quinidine-based monolithic column–Analysis of l-carnosine in dietary supplements, Journal of Chromatography A 1428 (2016) 176-184.

[128] Q. Zhang, V. Gil, E. Sánchez-López, M.Á. García, Z. Jiang, M.L. Marina, Evaluation of the potential of a quinidine-based monolithic column on the enantiomeric separation of herbicides by nano-liquid chromatography, Microchemical Journal 123 (2015) 15-21.

[129] H. Wu, Q. Wang, M. Ruan, K. Peng, P. Zhu, J. Crommen, H. Han, Z. Jiang, Enantioseparation of N-derivatized amino acids by micro-liquid chromatography/laser induced fluorescence detection using quinidine-based monolithic columns, Journal of pharmaceutical and biomedical analysis 121 (2016) 244-252.

[130] Q. Wang, P. Zhu, M. Ruan, H. Wu, K. Peng, H. Han, G.W. Somsen, J. Crommen, Z. Jiang, Chiral separation of acidic compounds using an O-9-(tert-butylcarbamoyl) quinidine functionalized monolith in micro-liquid chromatography, Journal of Chromatography A 1444 (2016) 64-73.

[131] B. Preinerstorfer, D. Lubda, A. Mucha, P. Kafarski, W. Lindner, M. Lämmerhofer, Stereoselective separations of chiral phosphinic acid pseudodipeptides by CEC using silica monoliths modified with an anion-exchange-type chiral selector, Electrophoresis 27 (2006) 4312-4320.

[132] D. Lubda, W. Lindner, Monolithic silica columns with chemically bonded tert-butylcarbamoylquinine chiral anion-exchanger selector as a stationary phase for enantiomer separations, Journal of Chromatography A 1036 (2004) 135-143.

[133] S. Buchinger, B. Follrich, M. Lämmerhofer, D. Lubda, W. Lindner, Chirally functionalized anion-exchange type silica monolith for enantiomer separation of 2-aryloxypropionic acid herbicides by non-aqueous capillary electrochromatography, Electrophoresis 30 (2009) 3804-3813.

[134] B. Preinerstorfer, D. Lubda, W. Lindner, M. Lämmerhofer, Monolithic silica-based capillary column with strong chiral cation-exchange type surface modification for enantioselective non-aqueous capillary electrochromatography, Journal of Chromatography A 1106 (2006) 94-105.

[135] B. Preinerstorfer, C. Hoffmann, D. Lubda, M. Lämmerhofer, W. Lindner, Enantioselective silica-based monoliths modified with a novel aminosulfonic acid-derived strong cation exchanger for electrically driven and pressure-driven capillary chromatography, Electrophoresis 29 (2008) 1626-1637.

[136] B. Preinerstorfer, M. Lämmerhofer, C.V. Hoffmann, D. Lubda, W. Lindner, Deconvolution of electrokinetic and chromatographic contributions to solute migration in stereoselective ion-exchange capillary electrochromatography on monolithic silica capillary columns, Journal of separation science 31 (2008) 3065-3078.

[137] L.N. Tran, J.H. Park, Enantiomer separation of acidic chiral compounds on a quinine-silica/zirconia hybrid monolith by capillary electrochromatography, Journal of Chromatography A 1396 (2015) 140-147.

[138] L.N. Tran, J.A. Jeong, J.H. Park, Enantiomer Separation of Acidic Chiral Compounds on a tert-Butylcarbamoylquinine-Silica Hybrid Monolith by Capillary Electrochromatography, Bulletin of the Korean Chemical Society 37 (2016) 1050-1056.

[139] S. Ma, Y. Wang, H. Zhang, Y. Li, J. Ou, Y. Wei, M. Ye, One-step fabrication of cinchona-based hybrid monolithic chiral stationary phases via photo-initiated thiol-ene polymerization for cLC enantioseparation, Talanta 198 (2019) 432-439.

[140] R. Däppen, H. Arm, V.R. Meyer, Applications and limitations of commercially available chiral stationary phases for high-performance liquid chromatography, Journal of Chromatography A 373 (1986) 1-20.

[141] M. Kato, M.T. Dulay, B. Bennett, J.r. Chen, R.N. Zare, Enantiomeric separation of amino acids and nonprotein amino acids using a particle-loaded monolithic column, ELECTROPHORESIS: An International Journal 21 (2000) 3145-3151.

[142] M.D. Slater, J.M. Fréchet, F. Svec, In-column preparation of a brush-type chiral stationary phase using click chemistry and a silica monolith, Journal of separation science 32 (2009) 21-28.

[143] R. Sancho, A. Novell, F. Svec, C. Minguillón, Monolithic silica columns functionalized with substituted polyproline-derived chiral selectors as chiral stationary phases for high-performance liquid chromatography, Journal of separation science 37 (2014) 2805-2813.

[144] A. Novell, C. Minguillón, Monolithic silica columns with covalently attached octaproline chiral selector. Dependence of performance on derivatization degree and comparison with a bead-based analogue, Journal of Chromatography A 1384 (2015) 124-132.

[145] A. Novell, A. Méndez, C. Minguillón, Effects of supercritical fluid chromatography conditions on enantioselectivity and performance of polyproline-derived chiral stationary phases, Journal of Chromatography A 1403 (2015) 138-143.

[146] A. Ghanem, T. Ikegami, N. Tanaka, New silica monolith bonded chiral (R)-γ butyrolactone for enantioselective micro high-performance liquid chromatography, Chirality 23 (2011) 887-890.

[147] M. Ruan, Q. Wang, H. Wu, Y. Wang, H. Han, Z. Jiang, Preparation and evaluation of tert-leucine derivative functionalized polymeric monoliths for micro-liquid chromatography, Electrophoresis 38 (2017) 3020-3028.

[148] Q. Cao, Y. Xu, F. Liu, F. Svec, J.M. Frechet, Polymer monoliths with exchangeable chemistries: use of gold nanoparticles as intermediate ligands for capillary columns with varying surface functionalities, Analytical chemistry 82 (2010) 7416-7421.

[149] L.N. Mu, Z.H. Wei, Z.S. Liu, Current trends in the development of molecularly imprinted polymers in CEC, Electrophoresis 36 (2015) 764-772.

[150] Y.P. Huang, Z.S. Liu, C. Zheng, R.Y. Gao, Recent developments of molecularly imprinted polymer in CEC, Electrophoresis 30 (2009) 155-162.

[151] Y.-P. Huang, C. Zheng, Z.-S. Liu, Molecularly imprinted polymers for the separation of organic compounds in capillary electrochromatography, Current Organic Chemistry 15 (2011) 1863-1870.

[152] C. Zheng, Y.P. Huang, Z.S. Liu, Recent developments and applications of molecularly imprinted monolithic column for HPLC and CEC, Journal of separation science 34 (2011) 1988-2002.

[153] C. Zheng, Y.-P. Huang, Z.-S. Liu, Synthesis and theoretical study of molecularly imprinted monoliths for HPLC, Analytical and bioanalytical chemistry 405 (2013) 2147-2161.

[154] M. Rutkowska, J. Płotka-Wasylka, C. Morrison, P.P. Wieczorek, J. Namieśnik, M. Marć, Application of molecularly imprinted polymers in analytical chiral separations and analysis, TrAC Trends in Analytical Chemistry 102 (2018) 91-102.

[155] H.F. Wang, Y.Z. Zhu, X.P. Yan, R.Y. Gao, J.Y. Zheng, A room temperature ionic liquid (RTIL)-mediated, non-hydrolytic sol–gel methodology to prepare molecularly imprinted, silica-based hybrid monoliths for chiral separation, Advanced Materials 18 (2006) 3266-3270.

[156] J. Yin, G. Yang, Y. Chen, Rapid and efficient chiral separation of nateglinide and its l-enantiomer on monolithic molecularly imprinted polymers, Journal of Chromatography A 1090 (2005) 68-75.

[157] J.X. He, G.z. Fang, Y.c. Yao, S. Wang, Preparation and characterization of molecularly imprinted silica monolith for screening sulfamethazine, Journal of separation science 33 (2010) 3263-3271.

[158] H.Y. Zong, X. Liu, Z.S. Liu, Y.P. Huang, Molecular crowding-based imprinted monolithic column for capillary electrochromatography, Electrophoresis 36 (2015) 818-824.

[159] J. Matsui, S. Goji, T. Murashima, D. Miyoshi, S. Komai, A. Shigeyasu, T. Kushida, T. Miyazawa, T. Yamada, K. Tamaki, Molecular imprinting under molecular crowding conditions: an aid to the synthesis of a high-capacity polymeric sorbent for triazine herbicides, Analytical chemistry 79 (2007) 1749-1757.

[160] X.-H. Wang, Q. Dong, L.-L. Ying, S.-S. Chi, Y.-H. Lan, Y.-P. Huang, Z.-S. Liu,

Enhancement of selective separation on molecularly imprinted monolith by molecular crowding agent, Analytical and bioanalytical chemistry 409 (2017) 201-211.

[161] J. Kang, D. Wistuba, V. Schurig, A silica monolithic column prepared by the sol-gel process for enantiomeric separation by capillary electrochromatography, Electrophoresis 23 (2002) 1116-1120.

[162] M.L. Hsieh, G.Y. Li, L.K. Chau, Y.S. Hon, Single-step approach to β-cyclodextrin-bonded silica as monolithic stationary phases for CEC, Journal of separation science 31 (2008) 1819-1827.

[163] C. Lv, Y. Liu, D. Mangelings, Y. Vander Heyden, Enantioselectivity of monolithic silica stationary phases immobilized with different concentrations cellulose tris (3, 5-dimethylphenylcarbamate), analyzed with different mobile phases in capillary electrochromatography, Electrophoresis 32 (2011) 2708-2717.

[164] R. Mallik, D.S. Hage, Affinity monolith chromatography, Journal of separation science 29 (2006) 1686-1704.

[165] M. Ye, H. Zou, Z. Liu, R. Wu, Z. Lei, J. Ni, Study of competitive binding of enantiomers to protein by affinity capillary electrochromatography, Journal of pharmaceutical and biomedical analysis 27 (2002) 651-660.

[166] Z. Liu, K. Otsuka, S. Terabe, M. Motokawa, N. Tanaka, Physically adsorbed chiral stationary phase of avidin on monolithic silica column for capillary electrochromatography and capillary liquid chromatography, Electrophoresis 23 (2002) 2973-2981.

[167] T. Hong, C. Chi, Y. Ji, Pepsin-modified chiral monolithic column for affinity capillary electrochromatography, Journal of separation science 37 (2014) 3377-3383.

[168] D. Xu, H. Shao, R. Luo, Q. Wang, E. Sánchez-López, S. Fanali, M.L. Marina, Z. Jiang, A facile and efficient single-step approach for the fabrication of vancomycin functionalized polymer-based monolith as chiral stationary phase for nano-liquid chromatography, Journal of Chromatography A 1557 (2018) 43-50.

[169] C. Gatschelhofer, M.G. Schmid, K. Schreiner, T.R. Pieber, F.M. Sinner, G. Gübitz, Enantioseparation of glycyl-dipeptides by CEC using particle-loaded monoliths prepared by ring-opening metathesis polymerization (ROMP), Journal of biochemical and biophysical methods 69 (2006) 67-77.

[170] D. Xu, Q. Wang, E. Sánchez-López, Z. Jiang, M.L. Marina, Preparation of an O-

[2-(methacryloyloxy)-ethylcarbamoyl]-10, 11-dihydroquinidine-silica hybrid monolithic column for the enantioseparation of amino acids by nano-liquid chromatography, Journal of Chromatography A 1593 (2019) 63-72.

[171] H. Hödl, J. Koidl, M.G. Schmid, G. Gübitz, Chiral resolution of tryptophan derivatives by CE using canine serum albumin and bovine serum albumin as chiral selectors, Electrophoresis 27 (2006) 4755-4762.

[172] R. Gotti, E. Calleri, G. Massolini, S. Furlanetto, V. Cavrini, Penicillin G acylase as chiral selector in CE using a pullulan-coated capillary, Electrophoresis 27 (2006) 4746-4754.

[173] M. Kato, H. Saruwatari, K. Sakai-Kato, T. Toyo'oka, Silica sol–gel/organic hybrid material for protein encapsulated column of capillary electrochromatography, Journal of Chromatography A 1044 (2004) 267-270.

[174] A. Ghanem, F.G. Adly, Y. Sokerik, N.Y. Antwi, M.A. Shenashen, S.A. El-Safty, Trimethyl-β-cyclodextrin-encapsulated monolithic capillary columns: Preparation, characterization and chiral nano-LC application, Talanta 169 (2017) 239-248.

[175] A. Fouad, M.S.A. Shaykoon, S.M. Ibrahim, S.M. El-Adl, A. Ghanem, Colistin Sulfate Chiral Stationary Phase for the Enantioselective Separation of Pharmaceuticals Using Organic Polymer Monolithic Capillary Chromatography, Molecules 24 (2019) 833.

[176] J. He, X. Wang, M. Morill, S.A. Shamsi, Amino acid bound surfactants: A new synthetic family of polymeric monoliths opening up possibilities for chiral separations in capillary electrochromatography, Analytical chemistry 84 (2012) 5236-5242.

[177] K. Sakai-Kato, M. Kato, T. Toyo'oka, On-line trypsin-encapsulated enzyme reactor by the sol− gel method integrated into capillary electrophoresis, Analytical chemistry 74 (2002) 2943-2949.

[178] R. Noel Echevarria, E.J. Carrasco-Correa, S. Keunchkarian, M. Reta, J.M. Herrero-Martinez, Photografted methacrylate-based monolithic columns coated with cellulose tris (3, 5-dimethylphenylcarbamate) for chiral separation in CEC, Journal of separation science 41 (2018) 1424-1432.

[179] J. Mohammad, Y.M. Li, M. El-Ahmad, K.i. Nakazato, G. Pettersson, S. Hjertéean, Chiral-recognition chromatography of β-blockers on continuous polymer beds with immobilized cellulase as enantioselective protein, Chirality 5 (1993) 464-470.

[180] K.M. Kacprzak, N.M. Maier, W. Lindner, Highly efficient immobilization of Cinchona alkaloid derivatives to silica gel via click chemistry, Tetrahedron letters 47 (2006) 8721-8726.

[181] K.M. Kacprzak, N.M. Maier, W. Lindner, Triazolo-linked cinchona alkaloid carbamate anion exchange-type chiral stationary phases: Synthesis by click chemistry and evaluation, Journal of Chromatography A 1218 (2011) 1452-1460.

[182] K.M. Kacprzak, W. Lindner, Novel Pirkle-type quinine 3, 5-dinitrophenylcarbamate chiral stationary phase implementing click chemistry, Journal of separation science 34 (2011) 2391-2396.

[183] S. Currivan, P. Jandera, Post-polymerization modifications of polymeric monolithic columns: a review, Chromatography 1 (2014) 24-53.

[184] C. Chi, W. Wang, Y. Ji, Preparation and evaluation of pepsin affinity organic polymer capillary monolithic column, Se pu= Chinese journal of chromatography 32 (2014) 791-797.

[185] E. Calleri, G. Massolini, D. Lubda, C. Temporini, F. Loiodice, G. Caccialanza, Evaluation of a monolithic epoxy silica support for penicillin G acylase immobilization, Journal of Chromatography A 1031 (2004) 93-100.

[186] M. Jonnada, Z. El Rassi, Poly (N-acryloxysuccinimide-co-ethylene glycol dimethacrylate) precursor monolith and its post polymerization modification with alkyl ligands, trypsin and lectins for reversed-phase chromatography, miniaturized enzyme reactors and lectin affinity chromatography, respectively, Electrophoresis 38 (2017) 2870-2879.

[187] J. Wang, X. Wang, J. Li, H. Lü, X. Lin, Z. Xie, Q. Zhang, Preparation of chiral monolithic column with covalently bonded cellulose and their application to rapid enantioseparation, Se pu= Chinese journal of chromatography 29 (2011) 1222-1229.

[188] R. Mallik, C. Wa, D.S. Hage, Development of sulfhydryl-reactive silica for protein immobilization in high-performance affinity chromatography, Analytical chemistry 79 (2007) 1411-1424.

[189] X. Han, Y. Xie, Q. Wu, S. Wu, The Effect of Monolith Properties on the Digestion Performance of Monolith-Based Immobilized Enzyme Microreactor, Journal of chromatographic science 57 (2018) 116-121.

[190] S. Jiang, Z. Zhang, L. Li, A one-step preparation method of monolithic enzyme

reactor for highly efficient sample preparation coupled to mass spectrometry-based proteomics studies, Journal of Chromatography A 1412 (2015) 75-81.

[191] M. Guerrouache, B. Carbonnier, C. Vidal-Madjar, M.-C. Millot, In situ functionalization of N-acryloxysuccinimide-based monolith for reversed-phase electrochromatography, Journal of Chromatography A 1149 (2007) 368-376.

[192] F. Svec, Y. Lv, Advances and recent trends in the field of monolithic columns for chromatography, Analytical chemistry 87 (2014) 250-273.

[193] K. Peng, Q. Wang, W. Chen, D. Xia, Z. Zhou, Y. Wang, Z. Jiang, F. Wu, Phosphatidic acid-functionalized monolithic stationary phase for reversed-phase/cation-exchange mixed mode chromatography, RSC Advances 6 (2016) 100891-100898.

[194] Q. Zhang, J. Guo, Y. Xiao, J. Crommen, Z. Jiang, Comparative evaluation of a one-pot strategy for the preparation of β-cyclodextrin-functionalized monoliths: Effect of the degree of amino substitution of β-cyclodextrin on the column performance, Journal of separation science 38 (2015) 1813-1821.

[195] C. Gu, S.A. Shamsi, Evaluation of a methacrylate-bonded cyclodextrins as a monolithic chiral stationary phase for capillary electrochromatography (CEC)-UV and CEC coupled to mass spectrometry, Electrophoresis 32 (2011) 2727-2737.

[196] W. Bragg, S.A. Shamsi, A novel positively charged achiral co-monomer for β-cyclodextrin monolithic stationary phase: Improved chiral separation of acidic compounds using capillary electrochromatography coupled to mass spectrometry, Journal of Chromatography A 1267 (2012) 144-155.

[197] Y. Li, H. Liang, R. Lv, C. Zhang, L. Zhang, H. Fu, W. Zhang, One-step preparation and properties of allyl-β-cyclodextrin chiral monolithic capillary electrochromatography column, Scientia Sinica Chimica 40 (2010) 1682-1687.

[198] Y.-j. LI, S. DING, R.-j. Lü, H.-m. DING, L. DENG, X.-g. HOU, Preparation and Evaluation of α-Alkenyl-β-cyclodextrin Monolithic Column for Capillary Electrochromatography [J], Journal of Instrumental Analysis 11 (2011).

[199] Y. Hayashi, Pot economy and one-pot synthesis, Chemical science 7 (2016) 866-880.

[200] J. Guo, Y. Xiao, Y. Lin, Q. Zhang, Y. Chang, J. Crommen, Z. Jiang, Influence of the linking spacer length and type on the enantioseparation ability of β-cyclodextrin

functionalized monoliths, Talanta 152 (2016) 259-268.

[201] M. Deng, S. Li, L. Cai, X. Guo, Preparation of a hydroxypropyl-β-cyclodextrin functionalized monolithic column by one-pot sequential reaction and its application for capillary electrochromatographic enantiomer separation, Journal of Chromatography A (2019).

[202] M. Deng, M. Li, Y. Zhao, Z. Jiang, X. Guo, A novel one-pot strategy to prepare β-cyclodextrin functionalized capillary monoliths for enantioseparation of basic drugs, Talanta 189 (2018) 458-466.

[203] R. Putri, L.W. Lim, T. Takeuchi, Separation of Dansyl Amino Acids in Capillary Liquid Chromatography Using Cyclodextrin-Bonded Chiral Monolithic Stationary Phases, G P Globalize research J. Chem. 2 (2018) 59-67.

[204] M. Wu, R.a. Wu, F. Wang, L. Ren, J. Dong, Z. Liu, H. Zou, "One-pot" process for fabrication of organic-silica hybrid monolithic capillary columns using organic monomer and alkoxysilane, Analytical chemistry 81 (2009) 3529-3536.

LIST OF PUBLICATIONS

1. **D. Xu**, H. Shao, R. Luo, Q. Wang, E. Sánchez-López, S. Fanali, M. L. Marina, Z. Jiang. A facile and efficient single-step approach for the fabrication of vancomycin functionalized polymer-based monolith as chiral stationary phase for nano-liquid chromatography. *J. Chromatogr. A, 2018, 1557, 43-50.*

2. **D. Xu**, Q. Wang, E. Sánchez-López, Z. Jiang, M. L. Marina. Preparation of an O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine-silica hybrid monolithic column for the enantioseparation of amino acids by nano-liquid chromatography. *J. Chromatogr. A, 2019, 1593, 63-72.*

3. **D. Xu**, E. Sánchez-López, Q. Wang, Z. Jiang, M. L. Marina. Enantioseparation of amino acids using an O-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine-silica hybrid monolithic column by nano-liquid chromatography. Determination of norvaline and tryptophan in food supplements. *J. Pharm. Anal., DOI:10.1016/j.pha.2019.10.001*.

4. **D. Xu**, R. Luo, M. L. Marina, Z. Jiang. Single-step fabrication of a teicoplanin functionalized organic-silica hybrid monolith for enantioseparation by nano-liquid chromatography, *submitted.*

5. J. Guo, Q. Wang, **D. Xu**, J. Crommen, Z. Jiang. Recent advances in preparation and applications of monolithic chiral stationary phases*, submitted.*

6. R. Luo, H. Han, J. Liu, **D. Xu**, S. Fanali, Z. Jiang. Preparation and application of teicoplanin functionalized polymeric monolith for enantioseparation of chiral drugs. *J. Pharm. Biomed. Anal., submitted.*

CURRICULUM VITAE

Curriculum Vitae -Dongsheng Xu

***Full Name*:** Dongsheng Xu

***Gender/Birth*:** Male

***Birth*:** 16/09/1988

***Birthplace*:** Hubei, P. R. China

***Nationality*:** Chinese

***Email*:** *Dongsheng.Xu@edu.uah.es*

***Education*:**

- **09/2016 ~ present** University of Alcalá (Spain)
  Ph. D - Prof. M. L. Marina
- **09/2018 ~ 09/2019** Jinan University (China)
  Ph. D - Prof. Z. Jiang
- **09/2012 ~ 07/2015** Guizhou University of Chinese Medicine (China)
  M. Sc. - Prof. Y. Gao
- **09/2008 ~ 07/2012** Wuhan University of Bioengineering (China)
  B. S

ACKNOWLEDGMENTS

From 2016 to 2019, the time flew and it soon came to the end of my PhD Thesis. Looking back over the past three years, there are so much fun and unforgettable things rooting in my heart.

First of all, I would like to thank my PhD supervisors Prof. Maria Luisa Marina and Prof. Zhengjin Jiang. They not only accepted me to give me the opportunity to continue my academic studies, but also spent a lot of time to guide my research project so that I can successfuly finish my PhD Thesis. They are not only knowledgeable researchers, but also important supervisors and friends in my life. I am very fortunate that I have the opportunity to study in their research groups. This experience will also be my most valuable asset. Thanks to Prof. Maria Luisa Marina for giving me all the help and guidance during my life in Spain, and also for her patience and tolerance. Thanks to Prof. Zhengjin Jiang for training me skills and knowledge during my life in China, he is the most rigorous supervisor, and at the same time, he is also my best friend.

I would like to thank Elena who is my first friend in Spain. Thanks to her for helping me in my doctoral program. At work, I remember that she helped me to check all the data and gave me the greatest help and guidance for the problems in my experiment every week. Without her help, my research would not have been carried out so smoothly. At the life, thanks to her for helping me to apply for the visa and residence, thanks to her for helping me to complete admission registration and to apply for dormitory.

I would like to thank Gloria for taking me to apply for my residence card from Alcalá de Henares to Madrid. Thanks for all the help she gave me during my experiment. Without her friendly help, my life would have been very difficult.

I would like to thank Nuria, Romy, Merichel, Estefanía and Raquel. Thanks for their warm care and company, resulting that every trip is full of joy. Thanks for all the help they provided me to make my life full of warmth. Thank you for the birthday greetings you sent to me, it really made me feel very cordial.

The time in Spain is undoubtedly my most memorable experience. Thanks for all my colleagues: Maider, Jesús, Isa, Esther, Samuel, Sandra, Natalia, etc. I miss the time with you, I miss the delicious pizza, cake, paella, Christmas dinner…

I also would like to thank my Chinese friends Chusheng Liu, Jingcai Wang and Haibin Li. They are not only my good friends in private, but also gave me great support in the experiment and taught me a lot of experimental skills. Thanks for their patience and help to solve all the problems occurred in my experiment.

Thanks for Huikai Shao, and Rongying Luo. Due to your help, I could successfully complete my first article.

Thanks to Family Mart. It always provides me my favorite beer 24 hours a day. Thanks to every friend who drink with me in the Family Mart: Chusheng Liu, Jingcai Wang, Liu Song, Xiao Liu, Rongrong Xu, Liang Lai, etc. they always accompanied me to share happiness and sadness.

Thanks to all my Chinese colleagues: Haibo Zhou, Jialiang Guo, Hai Han, Tingting Zhang, Qiqin Wang, Yumei Zhao, Meng Ruan, Lvhuan Wang, Yuanjing Lin, Hanying Jin, Hang Lin, Yanyan Zhang, Sifan Luo, Jingyi Jian, Kun Peng, Yutian Lei, Jia Liu, Lingjue Sun, Ruijie Liu, Shengfeng Huang, Yang Huang, Jiaming Wu, Zijun Bao, Rentao Tang, Hao Huang, Ziwei Hu, etc.

In addition, I would like to thank Jian Zou and Huang Zhao. Thanks for your companionship from my undergraduate to doctoral period. Thanks for their help in the analysis of the monomers structure of this doctoral thesis.

Thanks to my families. Their contribution is silent, but undoubtedly is the greatest. Without their support and understanding, I could not have completed my doctoral project. I am looking forward to show you my final work, I think that they will be proud of me.

Finally, I want to thank myself, always insisted, never gave up!