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ORIGINAL RESEARCH

Covalent anchoring of a cellulose per(phenyl carbamate) chiral selector onto silica gel through alkyne-azide *click* chemistry and its utilization in HPLC

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Abstract High-performance liquid chromatography is a powerful tool for enantioseparation, based on chiral stationary phases as separation media. Cellulose-based chiral selectors are among the most successful ones used for the preparation of chiral separation materials, exploiting the inherent chirality of the homopolymer. Compared to initial coating-type chiral stationary phases solely deposited onto silica as the chromatographic support, covalently immobilized selectors exhibit a significantly broader scope of applicable eluents, but appropriate synthetic strategies are still scarce. In this work, we present the application of the Cu(I)-catalyzed *Huisgen* alkyne-azide *click* reaction as a means to covalently immobilize a cellulose 3,5-dichlorophenyl carbamate-type chiral selector to a silica-based chromatographic support. Cellulose was first functionalized with 3,5-dichlorophenyl carbamate groups (DS = 2.35) and 4-propargyloxy-3,5-dichlorophenyl carbamate groups (propargyl carbamate DS = 0.45, overall DS = 2.80), then clicked to 3-azidopropyl-functionalized silica gel as the chromatographic support affording a 9 wt.% covalently functionalized chiral stationary phase. The chiral selector was comprehensively characterized

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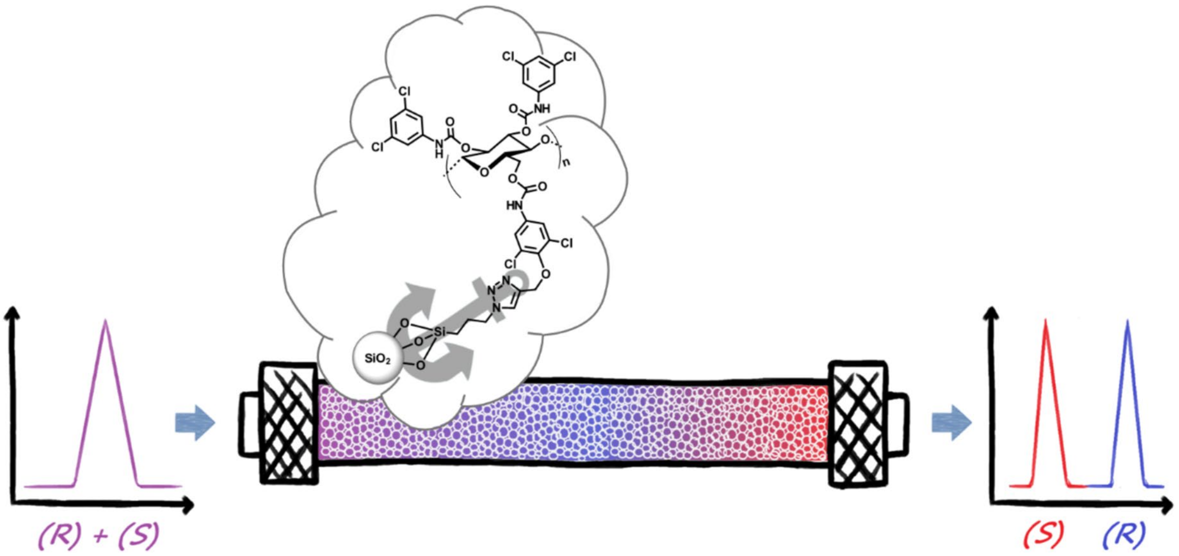
by means of ATR-FTIR and NMR spectroscopy, and elemental analysis. The degrees of substitution of both, overall functionalization and propargyl-anchor substitution, were estimated by NMR spectroscopy. Additionally, coating-type chiral stationary phases with 9 and 20 wt.% loadings were prepared. All chiral phases were tested with regard to their separation performance using a representative set of racemic analytes under usual normal-phase conditions. Solvent compatibility and thus the chemical robustness of the immobilized stationary phase was studied using higher shares of stronger, more polar solvents in the eluent, *i.e.*, ethyl acetate, tetrahydrofuran and chloroform. The covalently linked selectors performed very favorably with regard to separation performance and stability.

Graphical abstract



Keywords Cellulose · Chiral selector · *Click* chemistry · Enantioseparation · HPLC · Chiral liquid chromatography

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Introduction

Cellulose represents a readily available and affordable raw material for numerous industrial applications. Exhibiting convenient properties not only for large-scale use for example in paper and fiber/textile industries (Rosenau et al. 2019), several more specific utilizations on a lower scale, such as drug delivery, wound care (Sulaeva et al. 2020), tissue engineering, metal/dye removal, and chiral high-performance liquid chromatography (HPLC) are known (Bui et al. 2021; Dalei et al. 2022; Hesse & Hagel 1973; Hettegger et al. 2020). The latter application exploits the inherent chirality of cellulose, a polysaccharide consisting of β-(1→4)-linked D-glucopyranose units. Thus, cellulose is — by nature — a homochiral biopolymer and polysaccharide which can potentially be used to resolve chiral molecules by selective, stronger interaction with one of the two enantiomers, thereby increasing its retention time during chromatography. To enhance the separation performance, native cellulose is typically derivatized, thus improving non-bonding interactions with the analyte. At first, cellulose acetate was used as a chiral selector (CS) (Hesse and Hagel 1973; Lüttringhaus et al. 1967), while nowadays phenyl carbamates and esters are mostly used (Bui et al. 2021). Such cellulose and amylose derivatives represent standard selectors for separating low molecular weight chiral compounds in various fields of application, such as in the food and pharmaceutical industry, and pesticide production (Bui et al. 2023). Besides chiral low molecular weight compounds, nanoparticles (Dolamic et al. 2012) and metal cages (Izquierdo-García et al. 2023; Xue et al. 2023) exhibiting medium to high molecular weight were also separated by these polysaccharide-based CSPs. For utilization as separation materials in HPLC, the enantiopure CSs, *i.e.*, the cellulose derivatives, are combined with inert, mechanically stable carrier materials, mostly (functionalized) silica, to obtain chiral stationary phases (CSPs).

Due to their facile preparation, CSPs obtained by physical coating of the CS onto the chromatographic support have traditionally been the materials of choice (Okamoto et al. 1984). However, conditions under which coating-type CSPs can be operated are often limited by the comparably high solubility of the respective chiral selector in organic solvents, such as, *e.g.*, THF, acetone, or chloroform. This significantly restricts the choice of the mobile phase system, as the latter might cause leaching of the CS or even destruction of the column material through column bleeding, hampering resolution efficacy. To expand the applicable solvents and facilitate method optimization, covalent immobilization of the CS onto the silica material is often more convenient (Bui et al. 2021; Onishi et al. 2022). Several methods for the immobilization of cellulose-derived selectors have been reported to date. Cross-linking of cellulose 3,5-dimethylphenyl carbamate- and 3,5-dichlorophenyl carbamate selectors onto 3-aminopropyl-functionalized silica gel by diisocyanates was reported (Okamoto et al. 1987). However, the high degree of inter- and intramolecular linkages of the cellulose derivative and chromatographic support to some extent diminished the separation performance compared to their coating-type equivalents, due to the decreased flexibility of the CS (Chang et al. 2018; Chankvetadze 2013).

Following the pioneering work by Okamoto and co-workers, several new methods for the covalent immobilization of CSs have been developed to simultaneously maintain or even increase the separation performance with respect to their physically coated counterparts. Cross-linking with diisocyanates was advanced (Chen et al. 2002; Tang et al. 2011), while also vinyl group polymerization (Bae et al. 2011; Minguillón et al. 1996), alkoxysilyl condensation (Ikai et al. 2006; Tang et al. 2010), or photochemical immobilization of cellulose carbamate derivatives without additional linker agents (Francotte et al. 2016; Francotte and Huynh 2022) were reported as promising strategies. However, reactions of such self-cross-linking functional groups are rather hard to control. They can result in highly cross-linked networks of high rigidity and stiffness, often resulting in a diminished enantioseparation performance. Thus, better controllable chemistry, such as amidation (Han et al. 2017), implementation of epoxide-modified silica (Chankvetadze et al. 2004), Staudinger ligation (Lin et al. 2018; Zhang et al. 2007) or thiol-ene *click* chemistry (Huang et al. 2014; Zhou et al. 2020) are generally preferred, as they allow precise optimization of the number of linkages. Recently, we reported the implementation of another *click*-type reaction, the Cu(I)-catalyzed alkyne-azide *Huisgen* cycloaddition, to immobilize a cellulose 3,5-dimethylphenyl carbamate-type selector (Bui et al. 2023): azidopropyl-functionalized silica gel reacted with the CS which was equipped with propynyl groups at a low degree of substitution ($DS = 0.14$). While the viability of the approach was well confirmed, structural considerations raised questions about the appropriateness of the nature of the linker. The propynyl carbamate linker, as a rather short structural element, might limit the suppleness of the attached CS and thus diminish the overall separation performance. An increase in the length of this spacer group might not only allow for better accessibility of the reactive sites of the CS during the immobilization reaction but also increase the flexibility of the cellulose backbone chains during the separation process, possibly enhancing the material's performance when subjected to swelling/deswelling processes during changes in solvent composition.

In this work, we report the synthesis of cellulose-based CSs, functionalized with 3,5-dichlorophenyl carbamate, besides 4-propargyloxy-3,5-dichlorophenyl carbamate groups at a low degree of substitution ($DS_{anchor} = 0.45$; **CS1**). The CS was comprehensively characterized and, after either chemical immobilization or physical coating at different loadings, the resulting CSPs were packed into standard HPLC columns and tested using a representative set of chiral analytes to evaluate the separation performance under standard normal-phase (NP) conditions (*n*-hexane/*i*-PrOH 90:10, v/v). The good solvent compatibility of the immobilized stationary phase was further corroborated by using higher shares of more polar solvents with high elution power, such as EtOAc, THF, and $CHCl_3$.

Materials and methods

Materials

Microcrystalline cellulose (MCC, Avicel® PH-101), *N,N*-diisopropylethylamine (> 99%), *p*-toluenesulfonic acid (98.5%), tetra-*n*-butylammonium iodide (99%), *p*-cymene (99%), and 3,5-dichloro-4-hydroxybenzoic acid (97%) were purchased from Sigma-Aldrich (Schnelldorf, Germany) and used without further purification. MCC was dried at 40 °C in a vacuum oven for at least 2 d before use. 3,5-Dichlorophenyl isocyanate (> 98%), (3-chloropropyl)trimethoxysilane (> 97%) and NaN_3 (> 99%) were purchased from TCI Europe N.V. (Zwijndrecht, Belgium). Organic solvents, such as *N,N*-dimethylacetamide (DMAc), *N,N*-dimethylformamide (DMF), tetrahydrofuran (THF), acetonitrile (MeCN), and pyridine were all of reagent grade and dried over either 3 Å or 4 Å activated molecular sieves (Sigma-Aldrich) for at least 5 d before use. Acetone and methanol (MeOH) for precipitation and washing were of technical grade and obtained from Carl Roth GmbH + Co. KG (Karlsruhe, Germany) or Fisher Scientific (Vienna, Austria) and used as received. Silica gel (DAISOGEL, grade SP-300-5P, 5 µm, 300 Å, 115 m^2/g by BET) was purchased from Osaka Soda Co. Ltd. (Osaka, Japan). Empty stainless-steel HPLC columns (150 × 3.0 mm, i.d.) and column hardware were purchased from Bischoff Analysentechnik u. -geräte GmbH (Leonberg, Germany). The commercially available chiral analytes, 2-phenylcyclohexanone (> 98%, **A**), benzoin (> 98%, **B**), and Pirkle's alcohol (> 99%, **D**), were purchased from TCI Europe N.V. (Zwijndrecht, Belgium). Flavanone (98%, **C**), *trans*-stilbene oxide (98%, **E**), and Tröger's base (98%, **F**) were obtained from Sigma-Aldrich (Schnelldorf, Germany). The mandelic acid derivatives (**G**–**J**), 1-methoxy-2-(1-methoxy-3-phenylpropyl) benzene (**K**), 1-(*o*-hydroxyphenyl)-3-phenyl-1-propanol (**L**), and the ibuprofen derivatives (**M**–**P**) were synthesized according to standard procedures. The HPLC solvents *n*-hexane (HPLC grade, 95%; Carl Roth GmbH, Karlsruhe, Germany), 2-propanol (*i*-PrOH; HPLC grade, 99.9%; VWR, Vienna, Austria;), tetrahydrofuran (THF; 99.9%, non-stabilized; Carl Roth GmbH, Karlsruhe, Germany), and chloroform (for chiral analyte screening measurements: 99.8% stabilized with 1% ethanol; Sigma-Aldrich, Vienna, Austria; for stability test: HPLC grade, stabilized with amylene; TCI Europe N.V., Zwijndrecht, Belgium) were obtained commercially and used without further purification.

Instrumentation

Elemental analyses (EA) were performed on a EURO EA 3000 CHNS-O instrument (HEKAtech, Wegberg, Germany) at the Microanalytical Laboratory of the University of Vienna, Austria. Attenuated Total Reflection Fourier-Transformation Infrared (ATR-FTIR) spectra were recorded on a Frontier IR single-range spectrometer (PerkinElmer, Waltham, MA, USA) equipped with a diamond/ZnSe crystal, $LiTaO_3$ detector, and KBr windows. Data was obtained and processed using the PerkinElmer Spectrum software (version 10.03.02). The samples were lyophilized for a minimum of 24 h before the measurement. All spectra were obtained at a spectral range of 4000–600 cm^{-1}, a spectral resolution of 4 cm^{-1}, and 16 scans per sample. Thermogravimetric analysis (TGA) was carried out on a TG 209 F1 Iris thermomicrobalance (Netzsch GmbH & Co. KG, Selb, Germany), with a dried sample mass of 10–15 mg, an oxidizing atmosphere (N_2:O_2 = 4:1, v/v), a flow rate of 20 mL/min and a temperature gradient of 20 °C/min up to a maximum temperature of 1000 °C. Solution-state NMR spectra were recorded using a Bruker Avance II 400 spectrometer equipped with a cryogenically-cooled 5 mm broadband observe (BBO) probe-head with z gradients (CryoProbe™ Prodigy, N_2-cooled) (Bruker, Rheinstetten, Germany). The NMR experiments were performed at 303 K (unless indicated otherwise) at resonance frequencies of 400.13 MHz for ^{1}H and 100.61 MHz for ^{13}C using standard Bruker pulse programs. Chemical shifts are given in parts per million (ppm) and were referenced to the respective residual solvent signal as internal reference (DMSO-d_6: 2.50 ppm for ^{1}H, 39.52 ppm for ^{13}C). For all experiments, the number of scans and spectral widths were adjusted individually depending on the nature and the concentration of the sample. All NMR data were acquired and processed using Bruker TopSpin 3.2.7, 3.6.5, and/or 4.3.0 software. An Agilent Technologies, Inc. (Santa Clara, CA, USA) 1200 HPLC system equipped with a degasser (G1379B), binary pump (G1312B), autosampler (G1329B), thermostatted column compartment (G1316A), and DAD (G1315C) was used to evaluate the enantioseparation performance of the chiral columns. OpenLab CDS software (Agilent) was used for chromatography data processing and evaluation.

Syntheses

Synthesis of 4-propargyloxy-3,5-dichlorophenyl isocyanate (4) was carried out according to published procedures (Hettegger et al. 2014; Wolrab et al. 2013) implementing minor modifications. For details see Supporting Information (SI), section 1.1.

Synthesis of cellulose 3,5-dichlorophenyl and 4-propargyloxy-3,5-dichlorophenyl- carbamate (CS1): A two-necked round-bottom flask loaded with pre-dried MCC (0.300 g, 1.85 mmol, 1.0 equiv.) was flushed with N_2 for 1 h, before dry DMAc (9.0 mL, conc.: 33 g cellulose/L) was added, heated to 120 °C and vigorously stirred under a dry nitrogen atmosphere. After 2 h, the suspension was cooled to RT while anhydrous LiCl (0.547 g, 6.5% with respect to DMAc w/w) was slowly added during cooling at a temperature below 80 °C. The mixture was allowed to stir at RT until a clear solution had formed (approx. 3 h). The solution was heated to 80 °C and anhydrous pyridine (9.0 mL) was added. Subsequently, a solution of 3,5-dichlorophenyl isocyanate (0.349 g, 1.86 mmol, 1.0 equiv./AGU) in 1.0 mL of DMAc was added dropwise, whereupon the solution turned slightly yellow, and was stirred at 80 °C. After 8 h, a yellow solution of freshly synthesized 4-propargyloxy-3,5-dichlorophenyl isocyanate (0.249 g, 0,92 mmol 0.5 equiv.) in 2.0 mL anhydrous pyridine was added dropwise to the reaction mixture under stirring, whereupon gas evolution and increased viscosity were observed. After 12 h the clear reaction mixture had turned brown and a solution of 3,5-dichlorophenyl isocyanate (1.567 g, 8.33 mmol, 4.5 equiv./AGU) in 5 mL of anhydrous DMAc was added dropwise to the reaction mixture under inert atmosphere, whereupon the viscosity decreased. After another 24 h at 80 °C, the brown solution was poured into 300 mL of MeOH and the precipitate was collected by centrifugation. For purification, the precipitate was redissolved in 40 mL of acetone and reprecipitated in 200 mL of MeOH/H_2O (1:1, v/v). The solid product was collected by centrifugation, washed with diluted HCl ($M = 10^{-4}$ mol L^{-1}, pH = 4), deionized water, and subsequently freeze-dried to isolate the product as an off-white solid (yield = 1.35 g, quant.).

Pre-modification of silica gel (*AzPS*)

(a) **Synthesis of 3-chloropropyl-functionalized silica gel (ClPS)**: HPLC-grade silica gel (10 g) and *p*-toluene sulfonic acid (20 mg) as a catalyst were suspended in toluene (200 mL) and mechanically stirred. The suspension was dried by azeotropic distillation under an inert N_2 atmosphere using a Dean-Stark trap removing approx. 100 mL of solvent. The suspension was cooled to 80 °C, and (3-chloropropyl) trimethoxysilane (4.6 mL, 25.2 mmol) was added dropwise to the mixture over 15 min. The reaction mixture was mechanically stirred at this temperature for 38 h. Subsequently, the suspension was cooled down to RT and the product was collected by filtration through a glass frit funnel (DURAN®, porosity 4), and washed with toluene (2 × 100 mL) and MeOH (2 × 100 mL). The modified silica was re-suspended in toluene (100 mL), mechanically stirred at 80 °C for 2 h for further washing and cooled down to RT again. Purified ClPS was collected by vacuum filtration, washed with toluene (100 mL), MeOH (100 mL), and distilled water (200 mL, two times each), and dried at 40 °C in a vacuum oven for 2 d (**ClPS** yield = 9.97 g).

(b) **Synthesis of 3-azidopropyl-functionalized silica gel (AzPS)**: 3-Chloropropyl functionalized silica (9.97 g) was suspended in a 250 mL three-necked flask and 4.95 g of sodium azide (75.08 mmol) and 60 mg of tetra-*n*-butylammonium iodide in 100 mL of dimethyl sulfoxide (DMSO) were added. The reaction mixture was stirred for 3 d at 80 °C. The solid was filtered off with a sintered glass frit (porosity 4), and washed with 500 mL of deionized water, 250 mL of MeOH, and subsequently dried in a vacuum at 40 °C for 2 d. The azido-loading of **AzPS** was 289 µmol/g as determined by elemental analysis (calculated based on the N-content, for calculation see SI, Sect. 2.4).

Preparation of silica-immobilized CS (*CSP1*)

CS1 (0.60 g) was dissolved in THF (60 mL) in a Schott DURAN® bottle and continuously stirred. **AzPS** (2.5 g) was added and the suspension was shaken on a laboratory shaker at RT for 24 h. A catalyst solution was prepared by dissolving CuI (17 mg) in MeCN (10 mL). *N,N*-Diisopropylethylamine (1 mL) was added to the mixture, followed by the addition of the CuI solution. The mixture was degassed by purging with N_2 through a syringe needle for 30 min. The vessel was closed, sealed with Parafilm®, and allowed to slowly rotate for 24 h on an overhead shaker at RT. **CSP1** was collected by vacuum filtration through a sintered glass frit (DURAN®, porosity 4), washed with THF (200 mL), MeCN (100 mL), MeOH (100 mL), and distilled water (200 mL, two times each), and dried at 40 °C in a vacuum oven for 2 d (yield = 2.61 g, 84%).

Preparation of silica-coated CS (*CSP2*, *CSP3*)

A solution of the chiral selector (**CS1**) in THF (40 mL, HPLC grade, non-stabilized) was added to a flask loaded with **AzPS** in the desired weight ratio to obtain either 9 (**CSP2**) or 20 wt.% (**CSP3**) selector loading (see Table 1). The suspension was sonicated for 20 min, slowly evaporated to dryness at 40 °C (357 mbar), and further dried in the vacuum oven. Before column packing, the coated silica particles were sieved through an analytical sieve (40 µm mesh size). The loading was determined by TGA and EA analysis.

Table 1 Summarized data for the preparation of the coated CSPs

CSP	CS, loading [wt.%]	CS [g]	AzPS [g]	Yield (after sieving)
CSP2	CS1, 9%	0.270	2.73	2.73 g (91%)
CSP3	CS1, 20%	0.600	2.40	2.34 g (78%)

Column packing

For slurry packing, the respective CSP (1.2 g) was immersed in a mixture of *i*-PrOH (20 mL) and acetic acid (100 µL). The suspension was sonicated in an ultrasonic bath for 20 min to form a homogeneous slurry and left to settle for 10 min, after which the supernatant was decanted and the procedure was repeated. After the addition of fresh *i*-PrOH (20 mL) and acetic acid (100 µL), the suspension was sonicated, and the formed homogeneous slurry was packed in-house into a stainless steel HPLC column (column dimensions: 150 × 3.0 mm) using a high-pressure pump ECP2010H (ECOM, Prague, Czech Republic). The slurry was compacted at a constant pressure of 200 bar using MeOH (0.22 mL/min). After packing, the columns were rinsed with *i*-PrOH in a standard HPLC setup (flow rate: 1.0 mL/min) and stored until further use.

HPLC analyses

Before use, the columns were rinsed with *n*-hexane/*i*-PrOH using a stepwise gradient (30:70, 60:40, 90:10, v/v). The concentration of all analytes was 1.0 mg/mL. The flow rate and injection volume were set to 1.0 mL/min and 5 µL, respectively. The absorbance of all analytes was recorded at 254 nm. *p*-Cymene was used to determine the dead time (t_0) of the system.

Results and discussion

Synthesis of the CS

The chiral selector cellulose 3,5-dichlorophenyl carbamate, equipped with 4-propargyloxy-3,5-dichlorophenyl carbamate groups at a low degree of substitution (**CS1**) was targeted. The functional anchor groups were attached for immobilization onto 3-azidopropyl-functionalized silica as the chromatographic support by a *Huisgen*-type Cu(I)-catalyzed azide-alkyne *click* reaction to create the triazole-containing linker unit (see Fig. 1).

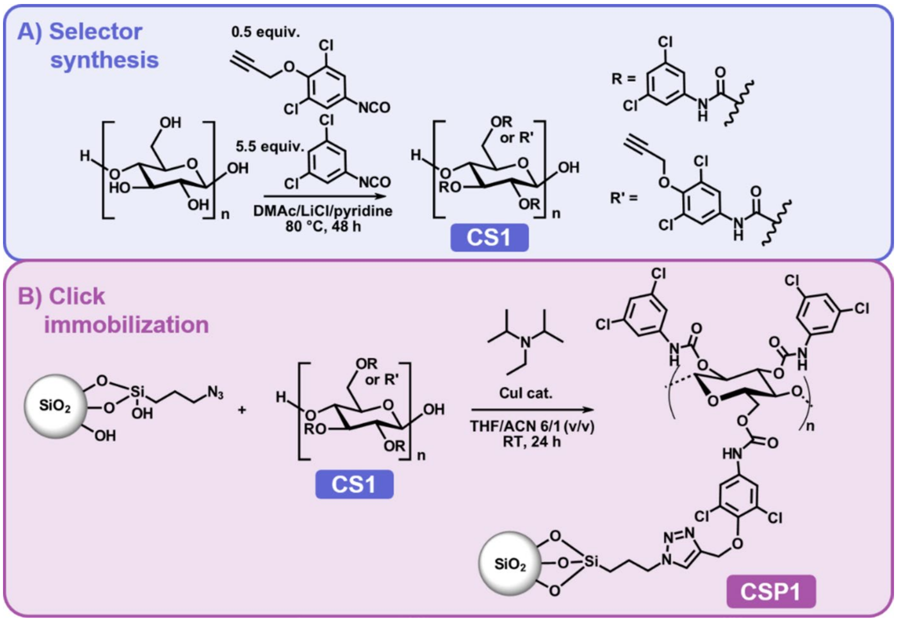

Fig. 1 Synthesis of **CS1** in a carbamoylation reaction (**A**) starting from Avicel® microcrystalline cellulose and immobilization of the latter via a Cu(I)-catalyzed Huisgen-type *click* reaction (**B**) onto N_3-functionalized silica gel, affording the immobilized **CSP1**

Functionalization of the starting material, Avicel® PH-101 cellulose, was carried out in solution to enable homogeneous distribution of the anchor groups along the cellulose chain in the product **CS1** (Fig. 1). The carbamoylation proceeded in a homogeneous DMAc/LiCl/pyridine (1/0.065/1) (v/w/v) medium. The pre-dried MCC was activated in anhydrous DMAc at 120 °C for 2 h, during which cellulose degradation was considered to be tolerable due to the absence of LiCl (Potthast et al. 2003). Before the addition of LiCl, the suspension was cooled to 80 °C or lower. To avoid the loss of the reactive anchor-containing isocyanate moiety due to hydrolytic side reactions from water traces, a 1.0 molar equivalent of 3,5-dichlorophenyl isocyanate (relative to AGU) was added initially to quench any residual water left in the reaction medium, before 0.5 equivalents of freshly synthesized 4-propargyloxy-3,5-dichlorophenyl isocyanate (**4**) were added. Subsequently, the remaining free OH groups were derivatized by an excess of 3,5-dichlorophenyl isocyanate. The resulting by-products were efficiently removed by sequential re-precipitation from acetone into a mixture of MeOH and H_2O (1:1, v/v) and through washing with diluted HCl and water.

The derivatization of cellulose with 3,5-dichlorophenyl isocyanate and 4-propargyloxy-3,5-dichlorophenyl isocyanate was confirmed by ATR-FTIR that showed a significant depletion of the OH band at 3000 cm^{-1} and an intense signal at 1721 cm^{-1} corresponding to the carbonyl group of the carbamates (ATR-FTIR spectra are provided in the SI). Further, the degree of substitution of **CS1** was estimated by quantitative ^1H NMR in DMSO-d_6 at 70 °C (Fig. 2). Elevated temperature measurements resulted in higher-quality spectra with well-resolved signals allowing integration, especially the separation of the carbohydrate region from the residual water signal. The integral of the protons corresponding to the saturated CH–O/CH_2–O groups of the glucopyranose repeating unit (3.5–5.5 ppm) was set to 7.0 resulting in an integral of 2.80 of the broad singlet at 10.65–9.00 ppm corresponding to the carbamate N–H protons (Fig. 2). Thus, NMR spectroscopic analysis suggests almost complete derivatization (DS = 2.80, 93%) of the polysaccharide backbone. Further, the DS of the anchor-containing moiety (4-propargyloxy-3,5-dichlorophenyl carbamate) was estimated by integration of the signals attributed to the alkyne proton (at $\delta_H = 3.37$, 3.42 and 3.45 ppm; three triplets possibly caused by three different regioisomers, sterically hindered rotation or even π–π-stacking of the aromatic rings; see Fig. 2, inset). By relating them to the CH–O/CH_2–O-sugar protons, a DS of 0.45 was calculated for the carbamate anchor unit, corresponding to a ratio of 16:84 between anchor and non-anchor

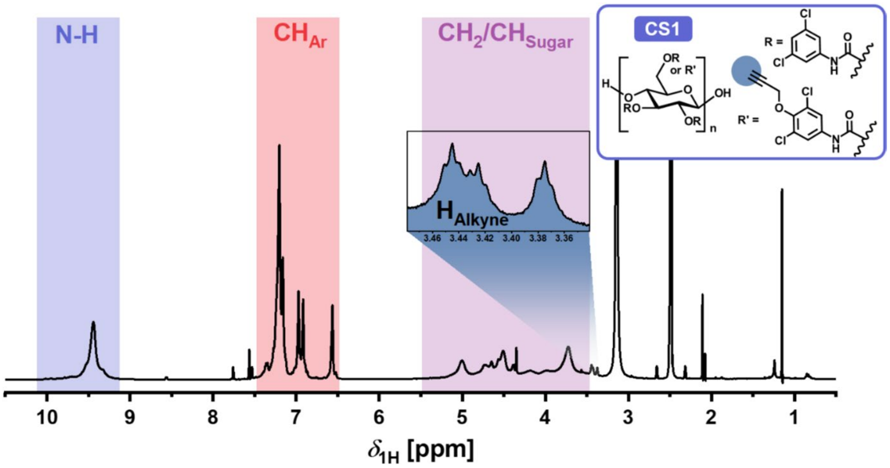

Fig. 2 ^{1}H NMR spectrum (DMSO-d_6 at 70 °C) of the mixed cellulose carbamate: 4-propargyloxy-3,5-dichlorophenyl ($DS_{anchor}=0.45$) 3,5-dichlorophenyl ($DS_{non\text{-}anchor}=2.35$) cellulose carbamate; inset: resonances of the terminal CH of the alkyne moiety

carbamate units (A:NA). Note that these calculations are only estimations, as the methylene (CH_2) signal from the anchor unit ($\delta_H=4.35$ ppm) lies within the CH-O/CH_2-O cellulose backbone spectral region and thus introduces an additional error. Furthermore, the overall DS of **CS1** was calculated based on elemental analysis (EA) data. Based on the A:NA ratio obtained from NMR spectroscopic analysis, the theoretical elemental composition was calculated for the maximum DS of 3.0 (see Table 2; detailed calculations are provided in the SI, section 2.4). The obtained nitrogen content was taken directly to quantify the carbamate substituents, leading to a calculated DS of 2.64 based on EA. Thus, near-quantitative functionalization of the CS with an actual DS of 2.64 to 2.80 (EA *vs.* ^{1}H NMR, respectively) can be assumed for **CS1**.

Functionalization of silica gel

Highly porous silica gel was derivatized according to a previously published procedure (Bui et al. 2023) and the loading was determined using TGA and EA (see Table 3). For the azidopropyl-functionalized silica (**AzPS**), the loading based on the residual dry mass was 3.64 wt.% (by TGA) and 4.69 wt.% (by EA). Based on the determined N-content (wt %, EA), a total N_3 content of 289 µmol/g was calculated.

Preparation of CSPs

The selector with propynyl functionalities (**CS1**) was chemically immobilized onto the N_3-functionalized **Az-PS** by means of a *Huisgen*-type *click*-reaction. The Cu(I)-catalyzed alkyne-azide cycloaddition proceeded smoothly under mild conditions in THF. The selector loading of the resulting **CSP1** was again determined by TGA and EA (see Table 3). In TGA, the total organic content of **CSP1** was determined. The mass loss due to the organic groups on the pre-modified silica gel (**AzPS**) was deducted from the overall mass loss, yielding a loading of 8.01% based on TGA (TGA curves see SI, section 2.5). Further, the loading was calculated based on the residual dry mass determined by EA. Yet again, the mass loss due to **AzPS** was subtracted leading to a total selector loading of 7.65%. The detailed calculation can be found in the SI, section 2.4.

Table 2 Elemental analysis data of the chiral selector **CS1**; overall DS calculated based on the N-content; DS_A (=degree of substitution of anchor carbamate units) and DS_{NA} (=degree of substitution of non-anchor carbamate units) were calculated based on the ratio of anchor and non-anchor units determined by ^{1}H NMR spectroscopy (16:84). *The Cl-content was calculated based on the difference between 100% and the sum of wt.% found for C, H, N and O

CS1	C [wt.%]	H [wt.%]	N [wt.%]	O [wt.%]	Cl [wt.%]	$DS_{overall}$	DS_A	DS_{NA}
theor	44.90	2. 651	5.721	17.77	28.96	-	-	-
exp	44.67 ± 0.04	2.88 ± 0.03	5.04 ± 0.03	19.92 ± 0.39	27.49 ± 0.39*	2.64	0.42	2.22

Table 3 Loading of the CS on different **CSPs** (**CSP1**-**CSP3**) calculated based on TGA and EA measurements; elemental analysis data of chiral stationary phases, mmol of functionalized anhydroglucose (AGU) unit per gram of CSP. Details on the calculations can be found in the SI, sections 2.4 and 2.5

Sample	Selector; type of loading (nominal loading)	Loading [%]		C [wt.%] (mmol/g)	H [wt.%] (mmol/g)	N [wt.%] (mmol/g)	Cl [wt.%] (mmol/g)	N_3-groups (µmol/g)
		TGA	EA					
Cl-PS	–	–	**4.62**	**1.38**	**0.45**	**n.d**	**0.96**	–
Az-PS	–	**3.65**	**4.69**	**1.46**	**0.41**	**1.21**	**0.05**	*288.8*
CSP1	**CS1**—clicked (9%)	**8.21**	**7.65**	**4.94** *(4.11)*	**0.54** *(5.44)*	**1.46** *(1.04)*	**2.21** *(0.63)*	–
CSP2	**CS1**—coated (9%)	**9.62**	**10.3**	**6.44** *(5.36)*	**0.75** *(7.42)*	**1.56** *(1.11)*	**2.37** *(0.66)*	–
CSP3	**CS1**—coated (20%)	**17.1**	**18.7**	**10.1** *(8.36)*	**0.97** *(9.62)*	**1.88** *(1.34)*	**4.56** *(1.28)*	–

Bold: wt.%; Not bold: mmol/g

Coating-type (not covalently bound) phases **CSP2** and **CSP3** were prepared by slow evaporation of a THF solution of **CS1** onto **AzPS**. CSPs exhibiting lower (~9 wt.%; **CSP2**) and higher loading (~20 wt.%; **CSP3**) were analyzed by TGA and EA as described above for **CSP1** (see Table 3). The loading difference from the nominal value presumably originates from insufficient drying of the components prior to coating and mechanical abrasion during sieving.

Enantioseparation performance

All three CSPs were tested under normal-phase (NP) HPLC conditions using the eluent *n*-hexane/*i*-PrOH 90:10 (v/v) at a flow rate of 1.0 mL min^{-1} with UV detection at 254 nm and a typical run time of 10 min. A representative set of chiral analytes was used for evaluating the CSPs' separation performance (Fig. 4). Under standard NP conditions, the performance of **CSP1** was superior compared to its coated counterpart **CSP2** (Fig. 5). The increase in selector loading

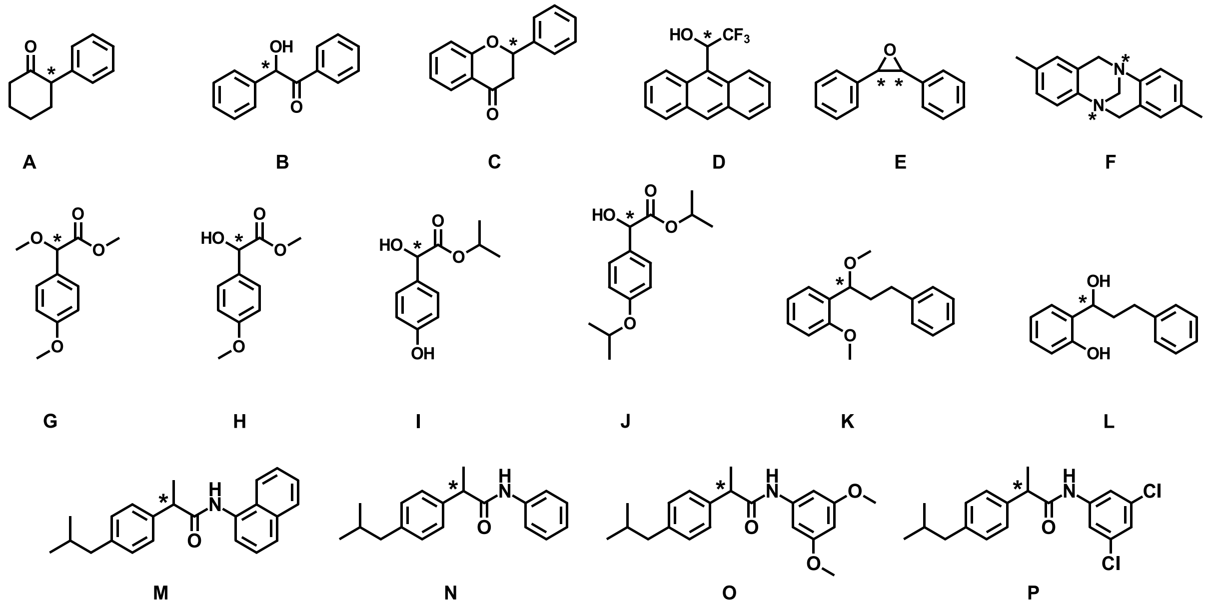

Fig. 4 Chemical structures of the chiral analytes **A-P**. Chirality centers are indicated with an asterisk

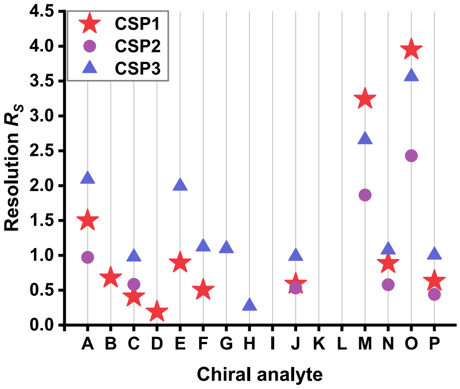

Fig. 5 Comparison of the resolution values (R_s) for the chiral analytes **A-P** on the chiral stationary phases **CSP1** (red stars), **CSP2** (purple circles), and **CSP3** (blue triangles) with *n*-hexane/*i*-PrOH 90:10 (v/v) as the mobile phase; when R_s was equal to 0, data is not visualized in the graph

from around 9 wt.% (**CSP2**) to 20 wt.% (**CSP3**) for the coating-type phases generally enhanced the separation performance. Analyte **A** was partially separated on **CSP2** ($R_s = 0.97$). However, baseline separation of the enantiomers was only achieved by using the clicked-type **CSP1** ($R_s = 1.50$) or **CSP3** with its higher loading ($R_s = 2.09$).

While **CSP1** was the only tested CSP capable of separating analytes **B** and **D**, the higher selector loading of **CSP3** enabled the separation of analytes **G** and **H**. Two of the ibuprofen derivatives, **M** and **O**, were also well-separated by **CSP2** ($R_s = 1.87$ and 2.43, respectively). The enantiomeric resolution of these analytes was enhanced by increased loading (**CSP3**; $R_s = 2.66$ and 3.56), but even further by implementing the *clicked*-type material **CSP1** ($R_s = 3.24$ and 3.95). While the higher resolution of **CSP3** compared to **CSP2** was accompanied by an almost two-fold increase of the retention time, **CSP1** exhibited higher efficiency regarding the separation of these enantiomers with a significantly higher number of theoretical plates at elution times comparable to **CSP2** (for detailed data on all analytes/CSPs see SI, section 3). We assume that this superior performance of **CSP1** is attributed to the covalent immobilization of the CS taking place in the solution state in THF. With the hierarchically chiral assembly of cellulose-based CSs being crucial for efficient enantioseparation (Lämmerhofer 2010), the slower and more controlled formation of the secondary structure would result in superior binding pockets in terms of well-ordered and more uniform structural arrangement (Chankvetadze 2013), which are accessible for chiral analytes. Further, the covalent linkage of the CS to the silica matrix via a sufficiently long anchor group — if possible at a low number of linkages — might improve the flexibility of the cellulose-backbone chains while simple coating just deposits the CSs on the carrier surface and thus restricts their mobility, adaptability, and ability to interact with the analyte molecules.

To evaluate the influence of higher elution strengths or additional mobile phase components on the separation performance of **CSP1**, all chiral analytes **A-P** were screened at different mobile phase compositions. Due to the covalent immobilization of the chiral selector on the chromatographic support, solvents with higher elution strength can be added to the mobile phase, which would normally — at least partially — dissolve the chiral selector and cause column bleeding for a coating-type CSP. For the screening experiments, EtOAc, THF, and $CHCl_3$ were used at 5, 10, and 20 vol.% each. Especially at low concentrations of the stronger eluents, the enantiomeric resolution was improved for some of the analytes compared to the standard mobile phase (Fig. 6a–c). In the case of analyte **O**, the addition of only 5 vol.% EtOAc or THF increased the resolution from 3.95 to 5.40 and 6.47, respectively. The addition of either 5 vol.% EtOAc or $CHCl_3$ significantly enhanced the separation of analyte **B**; in the case of 5 vol.% THF, even baseline separation was achieved (*cf.* Fig. 7, top left). For both analytes **D** and **L**, low or even no resolution of the enantiomers was accomplished with the standard mobile phase and the addition of EtOAc or THF. However, when $CHCl_3$ was added, the selectivity was enhanced, especially in the case of analyte **D** (Fig. 7). These results demonstrate that chemically immobilized chiral stationary phases permit much greater flexibility regarding the choice of the mobile phase. As shown, the addition of stronger eluents might improve or even enable the separation of enantiomers, which were not resolved using the standard mobile phase *n*-hexane/*i*-PrOH (90/10, v/v).

The stability of the immobilized **CSP1** was tested by constant column flushing with *n*-hexane/*i*-PrOH/$CHCl_3$ 79/1/20 (v/v/v) for a total of 33 h, injecting analyte **O** every 4 h. The chromatograms of the chiral

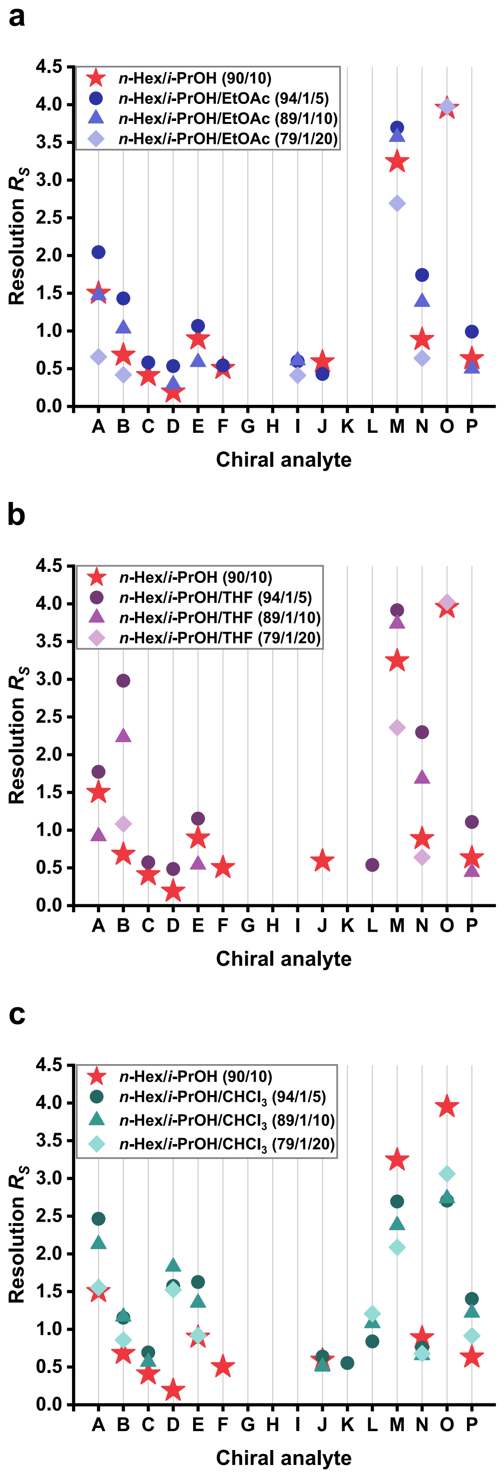

Fig. 6 Compatibility and performance of the immobilized ▸ stationary phase **CSP1** with mobile phases of higher elution strength, achieved by adding another solvent to the standard mobile phase *n*-hexane/*i*-PrOH 90:10 (v/v): **a**: 5, 10, and 20% of EtOAc; **b**: 5, 10, and 20% of THF; **c**: 5, 10, and 20% of $CHCl_3$. The resolution (R_s) for the chiral analytes **A**-**P** separated on **CSP1** is given for all tested mobile phases. In each graph, the standard mobile phase is given as a reference (red stars). The mobile phase compositions are given in volume ratios (v/v or v/v/v)

analyte **O** showed no significant change with regard to both retention times and enantioseparation performance (Figs. 6d and 8). This good retained separation performance is due to the covalent bonding of the CS to the chromatographic support: as already indicated, mobile phases containing chloroform or THF are prone to dissolve cellulose carbamate CSs in the case of coating-type materials and would thus significantly decrease — or in the worst case cancel — the separation performance already after short periods of flushing.

Conclusions and outlook

A cellulose 3,5-dichlorophenyl carbamate-type chiral selector was equipped with propynyl anchor groups at a low DS of approx. 0.45 in a stepwise carbamoylation using the respective isocyanates. The selector **CS1** was characterized by ATR-FTIR and NMR spectroscopy, and EA. Chemical immobilization of **CS1** onto 3-azidopropyl-functionalized silica gel in a *Huisgen*-type *click* reaction resulted in a selector loading of 9 wt.% (**CSP1**). For comparison, coating-type stationary phases of the same silica and selector lacking *click* immobilization were prepared with 9 and 20 wt.% loading. The selector loadings of all CSPs were determined using EA and TGA. The enantioseparation performances of the immobilized **CSP1** and its coated counterparts **CSP2** and **CSP 3** were evaluated using a set of 16 chiral analytes under standard NP conditions (*n*-hexane/*i*-PrOH 90/10, v/v). The immobilized **CSP1** generally showed superior separation performance compared to the coating-type **CSP2**. Higher loading in **CSP3** enhanced the resolution, however, it also led to a significant increase in retention time. Further, different eluents were tested with **CSP1** regarding their influence on the separation performance and stability of the stationary phase. The eluent screening demonstrated broad solvent compatibility of the immobilized CSP and further showed increased performance for specific analytes.

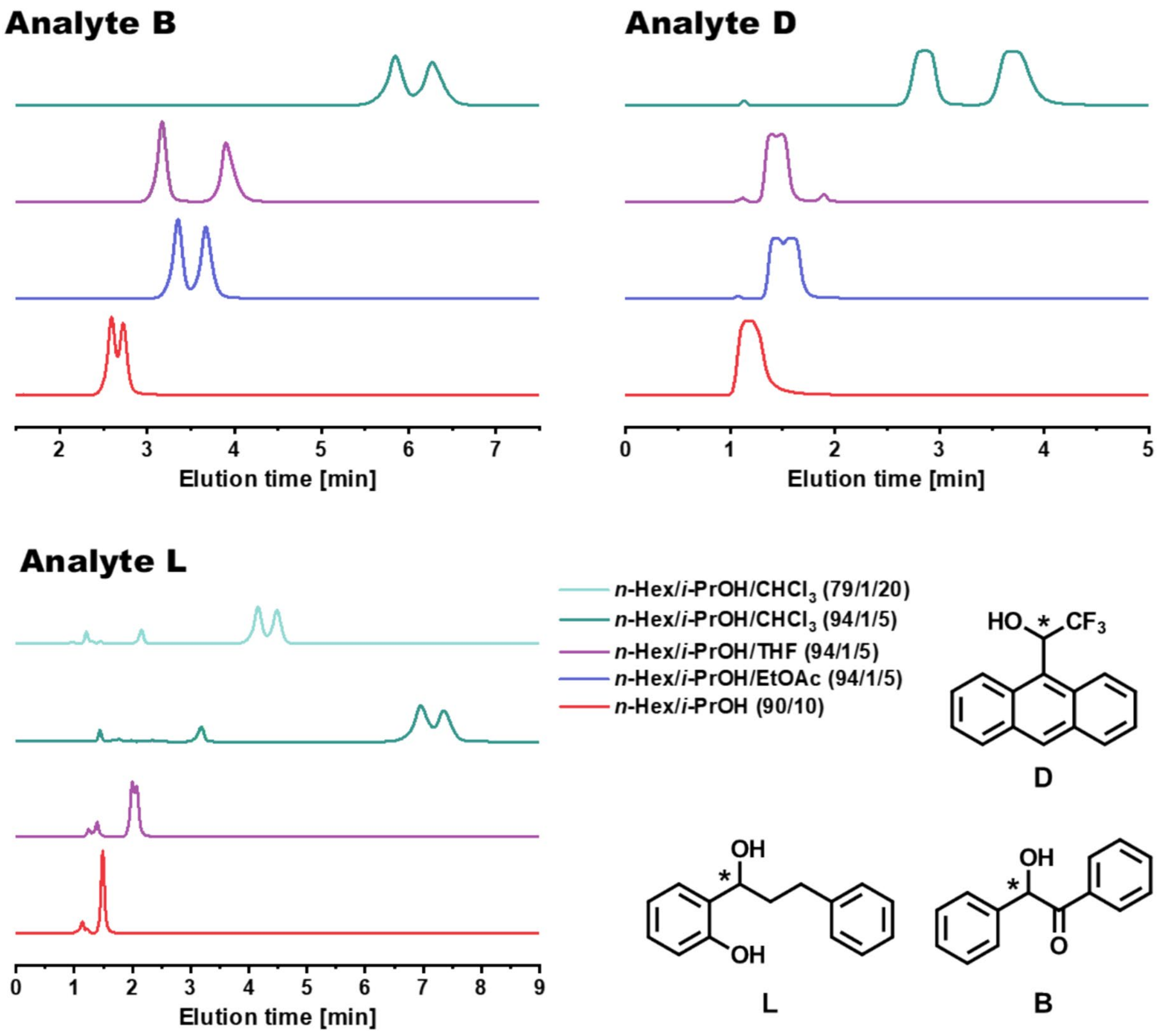

Fig. 7 Compatibility and performance of the immobilized stationary phase **CSP1** with mobile phases of higher elution strength: selected chromatograms of analytes **B**, **D**, and **L**. In each graph, the standard mobile phase *n*-hexane/*i*-PrOH (90/10, v/v) is given as a reference (red chromatogram). The mobile phase compositions are given in volume ratios (v/v or v/v/v)

A long-term stability test of **CSP1** with a strong-eluting mobile phase further proved to show only a minor decrease in separation performance. Thus, a broad range of different eluents—including those exhibiting high elution strength—can be used with the column material presented herein.

This work demonstrated the controlled synthesis of a per(phenylcarbamate)-type cellulose-based chiral selector covalently bonded to silica gel as the chromatographic support and its use as a CSP for HPLC applications. Our findings contribute to broadening the scope of immobilization methods available for

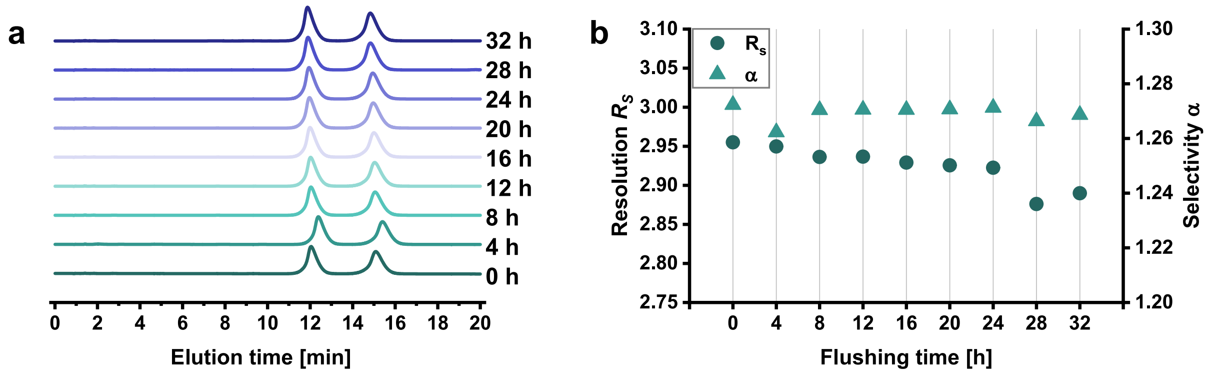

Fig. 8 Stability test of **CSP1** towards constant elution with *n*-hexane/*i*-PrOH/$CHCl_3$ (79/1/20, v/v/v) for 33 h; for evaluation. Analyte **O** was injected every 4 h; **a**: stacked chromatograms of the consecutive injections; **b**: resolution (R_s) and selectivity (α) values of the separations

polysaccharide-based chiral selectors and available immobilized chiral stationary phases.

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Data availability Data and materials are available from the authors upon request. No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Ethics approval and consent to participate Not applicable.

Consent for publication All authors agreed to the publication in the submitted form.

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