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New composite stationary phase for chiral high-performance liquid chromatography

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Abstract
The new composite chiral stationary phase for high-performance liquid chromatography was prepared and characterized. Poly(styrene-divinylbenzene) microspherical particles with diameter of 3.3 micron were used as a matrix and coated with layer of 10 nm gold nanoparticles. Immobilized gold nanoparticles increased specific surface area of adsorbent and simplify covalent attachment of sulfur-containing compounds. In this work L-lysine conjugate with lipoic acid was synthesized, characterized and used for modification of gold nanoparticles. The prepared chiral selector was immobilized by the reaction of sulfur-containing groups from lipoic acid residue with gold surface of nanoparticles with formation of self-assembled monolayer. The prepared chiral stationary phase was characterized by nitrogen adsorption at low temperatures, diffuse reflection spectroscopy, scanning electron microscopy. The chromatographic retention of beta-blockers and profens was studied under conditions of reversed-phase HPLC. The possibility of enantiomers separation was demonstrated for flurbiprofen and ketoprofen racemates using 100 × 4.6 mm ID chromatographic column.

Keywords Chiral stationary phases · Gold nanoparticles · Enantiomers · Poly(styrene-divinylbenzene) · HPLC

1 Introduction

It is well known that enantiomers of many essential drugs have different pharmaceutical activity. At present, many drugs having chiral center/s are marketed as a single pharmaceutically active enantiomer, but preparation of pure optical isomers is not a trivial task. For this reason, the separation of enantiomers is an important part of organic synthesis and medicinal chemistry. The most common solution of this problem is high-performance liquid chromatography (HPLC), which is widely used for separation and quantitative analysis of various classes of organic compounds including optical isomers. In the latter case, the successful separation of enantiomers requires the use of chromatographic systems containing, so called, chiral selectors either in mobile or in stationary phase. Obviously, to minimize the use of expensive chiral selectors it is more practical to use them in immobilized form. The corresponding adsorbents are called chiral stationary phases (CSP) and the corresponding separation mode got the name of chiral HPLC. CSPs for chiral HPLC can be classified according to the type of chiral selector as natural (chiral selectors are proteins, alkaloids, oligosaccharides, antibiotics), semi-synthetic (modified oligosaccharides, polysaccharides, low molecular weight natural compounds) and synthetic (polymers, low molecular weight synthetic compounds). There is no universal CSP allowing separation all possible enantiomers of different drugs, so there are many various types of stationary phases designed or attuned on separation of specific enantiomers. Consequently, there is a strong demand for the new selective CSPs, but as a rule, the synthesis of new CSPs is a complex research task, especially at the stage of immobilization of chiral selectors on the surface of an appropriate substrate.

One of the advanced methods for the preparation of efficient HPLC stationary phases is based on using nanoparticles [1] including metals nanoparticles (MNPs). MNPs, normally gold nanoparticles (AuNPs) as the most chemically inert nanoparticles can be easily prepared in various sizes and shapes by the reduction of Au(III) salts [2]. Traditionally, Turkevich's method using sodium citrate reduction of $HAuCl_4$ is used for the preparation of spherical AuNPs

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having various sizes [3]. The immobilized of AuNPs onto the surface of modified silica and organic polymers resulted in an increased surface area and formation of gold surface, which can be used for further modification with sulfur-containing ligands. In particular, the self-assembled reactions of thiols with gold surfaces are widely used for preparation of chemical sensors [4] and biosensors [5–7], flow through reactors [8] etc. The corresponding AuNPs have been also used in spectroscopic analysis [9–11]. Additionally, several applications of silica particles coated with MNPs as a stationary phase for liquid chromatography have been reported [1, 12–14]. The application of poly(styrene-divinylbenzene) (PS-DVB) microparticles containing AuNPs functionalized with *n*-octadecanethiol, thiophenol and 2-phenylethanethiol for separation of aromatic compounds has been also reported [15].

In this work, new method for preparation of CSP is proposed. The chiral selector is prepared by reaction of lipoic acid (LA) with L-lysine (Lys) followed by its selective adsorption onto surface of AuNPs coated PS-DVB microparticles via sulfur-containing group. The properties of obtained PS-DVB-Au-LA-Lys stationary phase were characterized and examined for the separation of enantiomers.

2 Experimental details

2.1 Reagents

All the reactions were carried out using freshly distilled solvents from solvent stills. Deionized water was used in all experiments. Reagents: N_{α}-Boc-L-lysine, (Sigma Aldrich, ≥ 99%), (±)-α-lipoic acid (Sigma Aldrich, ≥ 98.0%), *N*-hydroxysuccinimide (Alfa Aesar, ≥ 98%), $HAuCl_4 \cdot 3H_2O$ (Au-%, 50, ChemPur), sodium citrate tribasic dihydrate (Sigma Aldrich, ≥ 99%), CF_3COOH (ABCR, ≥ 99%), DCC (Sigma Aldrich, ≥ 99%), poly(styrene-divinylbenzene) spherical microparticles were synthesized by Dr. Pirogov A.V. (Department of Chemistry, Moscow State University), with the following characteristics: degree of crosslinking 50%, particle size 3.3 ± 0.2 μm, specific surface area 301 m^2/g, total pore volume 0.53 cm^3/g, average pore diameter 6.5 nm. Liquid chromatography on column packed with silica gel 60 (230–400 mesh) was used for isolation of pure Boc-derivative after amidation reaction.

2.2 Instrumentation

NMR spectra were acquired on Bruker Avance 500 spectrometer at room temperature; chemical shifts δ were measured in ppm with respect to solvent (CD_3OD: δH = 3.31 ppm; δC = 49.0 ppm). Splitting patterns are designated as t, triplet; m, multiplet; br.s., broad singlet. The structures of the synthesized compounds were elucidated with the aid of 1D NMR (1 H, 13C) and 2D NMR (HSQC) spectroscopy. IR spectra were recorded on Thermo Nicolet IR 200 FT-IR spectrometer. Registration of spectra was carried out at a resolution of 4 cm^{-1}, the number of scans 20. Samples were placed on the working surface of the diamond internal reflection (ATR) element with the angle of incidence of 45°. High-resolution mass spectra (HRMS ESI-TOF) were recorded on a Bruker microTOF-QTM spectrometer with electrospray ionization (ESI). The concentrations of Au were measured by microwave coupled plasma atomic emission spectrometry (Agilent 4200 MP-AES, USA) using the standard samples in 0.1 mg ml^{-1} concentration range for calibration. Scanning electron microscopy (SEM) images were obtained by using JEOL JCM-6000 (JEOL, Japan) instrument. Ci7800 benchtop sphere spectrophotometer (X-Rite, US) was used to obtain diffuse reflection spectra. Low-temperature nitrogen adsorption instrument ASAP Model 2020 (Micromeritics, Norcross, GA, USA) was used for characterization of porous structure of the prepared CSP. HPLC System LC-20 Prominence with photodiode array detector SPD-M20A (Shimadzu, Japan) was used for chromatography.

2.3 Preparation of CSP

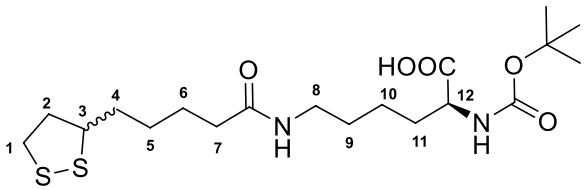
2.3.1 Synthesis of AuNPs

The solution of $HAuCl_4 \cdot 3H_2O$ (75 mg, 0.2 mmol) in water (110 ml) was heated to 100 °C. 1% solution of sodium citrate (26.25 ml) was added quickly under stirring of the reaction mixture at 100 °C. The heating was continued for 1 h until cherry color solution moved to the dark tones. The reaction mixture was cooled to room temperature. The resulting stable suspension of AuNPs was used for further experiments.

2.3.2 Absorption of AuNPs on the surface of PS-DVB particles

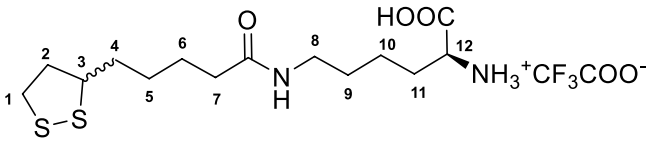
4 grams of PS-DVB microparticles were added to 80 mL of aqueous solution of 10 nm citrate stabilized AuNPs and obtained suspension was stirred at room temperature. After 12 h, prepared PS-DVB-Au particles were filtered and washed with water on a filter. The modification procedure was repeated four times. The resulting product was isolated as violet solid powder.

2.3.3 Synthesis of Boc-protected conjugate of (±)-α-lipoic acid and N_α-Boc-L-lysine



DCC (0.21 g, 1.02 mmol) was added to a solution of (±)-α-lipoic acid (0.20 g, 0.97 mmol) and *N*-hydroxysuccinimide (0.11 g, 0.97 mmol) in acetonitrile (5.0 ml) at room temperature. After being stirred for 1.5 h, the formed precipitate was filtered off and the filtrate was evaporated under reduced pressure. The resulting yellow oil of crude NHS ester (0.3 g, 1 mmol) was dissolved in dioxane-PBS, 3:1 (21 ml) and N_α-Boc-Lys-OH (0.27 g, 1.1 mmol) was added. After 12 h, 1 M HCl (2 ml) was added to a mixture and then it was extracted with DCM. Combined organic layers was dried over Na_2SO_4 and concentrated under vacuum. The residue was purified by silica gel column chromatography (DCM:MeOH = 100:1 to 10:1, $R_f = 0.50$) to give **1** (213 mg, 48%, mixture of two diastereomers, A: major diastereomer, B: minor diastereomer, A:B = 75:25) as a yellow oil (Table 1).

2.3.4 Boc-deprotection–formation of chiral selector N^6-(5-(1,2-dithiolan-3-yl)pentanoyl)-L-lysine trifluoroacetate (2)



1 mL of CF_3COOH was added to a solution of **1** (200 mg, 0.46 mmol) in 1 mL of DCM. The mixture was stirred at room temperature. After 1 h, the reaction mixture was evaporated under vacuum and residue was re-evaporated after addition of Et_2O to produce very viscous yellow oil (200 mg, 97%,). The resulting product representing a mixture of two diastereomers (A:B = 75:25) was used without further purification (Table 2).

Table 1 ^1H and ^{13}C NMR, IR, HRMS spectra of Boc-derivative 1

N^6-(5-(1,2-dithiolan-3-yl)pentanoyl)-N^2-(tert-butoxycarbonyl)-L-lysine (1)	
^1H NMR CD_3OD, 500 MHz	δ = 1.28–1.33 (m, 2H, CH_2, **A** + **B**, 10), 1.42–1.49 (br.s, 2H, CH_2, **A** + **B**, 5), 1.52–1.67 (m, 2H, **A**, 9 + 2H, **A** + **B**, 6 + 2H, **A** + **B**, 4), 1.69–1.79 (br.s, 2H, **B**, 9), 1.80–1.89 (m, 1H, CH_2, **A** + **B**, 2), 2.08–2.23 (m, 2H, **A** + **B**, 7), 2.34–2.44 (m, 1H, CH_2, **A** + **B**, 2), 2.62–2.65 (br.s, 2H, CH_2, **A**, 8), 2.66–2.68 (br.s, 2H, CH_2, **B**, 8), 3.00–3.08 (m, 2H, CH_2, **B**), 3.08–3.16 (m, 2H, CH_2, **A**), 3.46–3.52 (m, 1H, CH, **A**, **B**, 3), 3.93–4.04 (br.s, 1H, CH, **B**, 12), 4.06–4.17 (br.s, 1H, CH, **A**, 12)
^{13}C NMR CD_3OD 125 MHz	δ = 22.3 (CH_2, 10), 25.1 (CH_2, **A** + **B**, 6), 25.2 (CH_2, **A** + **B**, 9), 28.1 (3 × CH_3, **A** + **B**) 28.5 (CH_2, **B**, 5), 28.6 (CH_2, **A**, 5), 31.6 (CH_2, **A**, 11), 31.9 (CH_2, **B**, 11), 34.3 (CH_2, 4), 35.9 (CH_2, 7), 38.2 (C), 38.8 (CH_2, **A**, 1), 39.0 (CH_2, **B**, 1), 40.0 (CH_2, **A** + **B**, 2), 53.0 (CH, **A** + **B**, 12) 56.2 (CH, **A** + **B**, 3), 172.8 (COOH), 173.8 (CONH), 175.0 (CON, Boc)
IR ATR, cm^{-1}	3338 (ν_{OH}, ν_{HH}, ν_{NH}), 2973 (ν_{CH}), 2932 (ν_{CH}), 2862 (ν_{CH}), 1715 ($\nu_{C=O}$, carboxylic group), 1651 ($\nu_{C=O,\ amide}$), 1456 ($\delta CH_2 \delta CH_3$), 1391 ($\delta CH_2 \delta CH_3$), 1367 ($\delta CH_2 \delta CH_3$), 1213 (δ_{C-O-C}), 1168 (δ_{C-O-C})
HRMS ESI-TOF	m/z = 457.1801 $[M + Na]^+$ (457.1801 calcd for $C_{19}H_{34}N_2NaO_5S_2^+$)

Table 2 ^1H and ^{13}C NMR, IR, HRMS spectra chiral selector 2

N^6-(5-(1,2-dithiolan-3-yl)pentanoyl)-L-lysine trifluoroacetate (2)	
^1H NMR CD_3OD 500 MHz	δ = 1.45–1.74 (m, 10H, CH_2(4), CH_2(5), CH_2(6), CH_2(8), CH_2(10), **A** + **B**), 1.86–2.05 (m, 3H, CH(2), CH_2(11), **A** + **B**), 2.18–2.26 (m, 2H, CH_2(7), **A** + **B**), 2.30–2.42 (m, 1H, CH(2), **B**), 2.30–2.42 (m, 1H, CH(2), **A**), 3.08–3.24 (m, 4H, CH_2(1), CH_2(9), **A** + **B**), 3.42–3.49 (m, 1H, CH(3), **B**), 3.55–3.63 (m, 1H, CH(3), **A**), 3.97 (t, 1H, CH(12), **A** + **B**)
^{13}C NMR CD_3OD 125 MHz	δ = 21.5 (CH_2, **A** + **B**), 24.8 (CH_2, **A** + **B**), 27.95 (CH_2, **A**), 28.02 (CH_2, **B**), 29.3 (CH_2(11), **A** + **B**), 33.8 (2 × CH_2, **A** + **B**), 35.0 (CH_2, **A** + **B**), 37.5 (CH_2(1), CH_2(9), **B**), 37.9 (CH_2(1), CH_2(9), **A**), 39.4 (CH_2(2), **A** + **B**), 52.0 (CH(12)), 55.7 (CH(3)), 170.0 (COOH), 174.2 (CON), $\underline{C}F_3COO$ and $CF_3\underline{C}OO$ not observed.
IR ATR, cm^{-1}	3327 (ν_{OH}, ν_{HH}, ν_{NH}), 2933 (ν_{CH}), 2862 (ν_{CH}), 1927 ($\nu CF_3\boldsymbol{C(O)}O$), 1712 ($\nu_{C=O,}$ carboxylic group), 1628 ($\nu_{C=O,\ amide}$), 1202 (ν_{CF}).
HRMS ESI-TOF	m/z = 335.1451 $[M + H]^+$ (335.1458 calcd for $C_{14}H_{27}N_2O_3S_2^+$)

2.3.5 Immobilization of chiral selector on PS-DVB-Au

PS-DVB-Au particles (2.1 g) were added to a stirred water solution (70 ml) of **2** (310 mg) at room temperature in one portion. The reaction mixture was stirred at room temperature for 12 h. After reaction, the solid was filtered off and washed on the filter with water and MeOH and then dried on air.

3 Results and discussion

In this work, the CSP was prepared by immobilization a chiral selector onto the surface of gold nanoparticles coated PS-DVB microparticles. For this purpose, a chiral selector **2** representing adduct of lipoic acid and L-lysine was prepared as shown in Fig. 1. On the one hand, the lipoic acid contains a neutral sulfur-containing fragment, which is responsible for strong adhesion of chiral selector on gold nanoparticles in CSP by formation of Au-S covalent bonds. On the other hand, optically active L-lysine residue should provide chiral recognition of separated optical isomers of drugs. A peptide bond formation between carboxylic group in the molecule of lipoic acid and ω-amino groups in L-lysine is an optimal way for the preparation of target chiral selector **2**. To bond the right functional groups an established peptide synthesis procedure using N_α-Boc-protected (*tret*-butoxycarbonyl protecting group) L-lysine was applied. *N*-hydroxysuccinimide was used for activation of carboxylic group in lipoic acid molecule as previously described [16]. The obtained conjugate **1** was purified by silica gel column chromatography followed by removal of N_α-Boc-group by reaction with trifluoroacetic acid in CH_2Cl_2 to obtain **2** (Fig. 1). Product **2** was used for further experiments without purification. The conjugates were characterized by HRMS ESI-TOF and NMR and IR spectroscopy data.

10 nm citrate stabilized AuNPs were synthesized and used for coating PS-DVB particles as described [17] to obtain a stable suspension of PS-DVB-Au. Successful AuNPs adsorption was confirmed by atomic emission spectroscopy showing the presence 2.16 mass % gold in the PS-DVB matrix. The mass of a single AuNP is equal to 1.0×10^{-17} g by assuming average particle size 10 nm and metal gold density 19.0 g cm^{-3}. Hence, the resulting concentration of AuNPs immobilized onto PS-DVB matrix having specific surface area of 301 m^2 g^{-1} is approximately 2.17×10^{15} particles per gram.

Compounds **2** was coated onto a PS-DVB-Au chromatographic support by intensive stirring suspension PS-DVB-Au support in aqueous solution of **2** (Fig. 2). There is no clear evidence in the literature regarding breakage of the disulfide bonds within the molecule of lipoic acid, especially, in absence of strong reducing agents such as $NaBH_4$. On the contrary, the possibility of the formation of disulfide bonds between two adjacent thiol-containing ligands within self-assembled monolayers of thiols has been intensively discussed [18]. Anyway, Lin et al. [19] compared stability of gold nanoparticles treated with various carboxyl-terminated thiols and disulfides including lipoic acid and found that lipoic acid containing SAM is significantly more stable than those formed by monothiol-containing ligands [19]. According to the literature data the immobilisation of lipoic acid on gold surface both in presence (dithiolate form) [20] and in absence of $NaBH_4$ (disulfide form) [19] occurs at room temperature. Chiral selector adsorption has been proven by elemental analysis data. The result of the elemental analysis was 0.99% S that corresponded to 0.16 mmole g^{-1} of immobilized chiral selector.

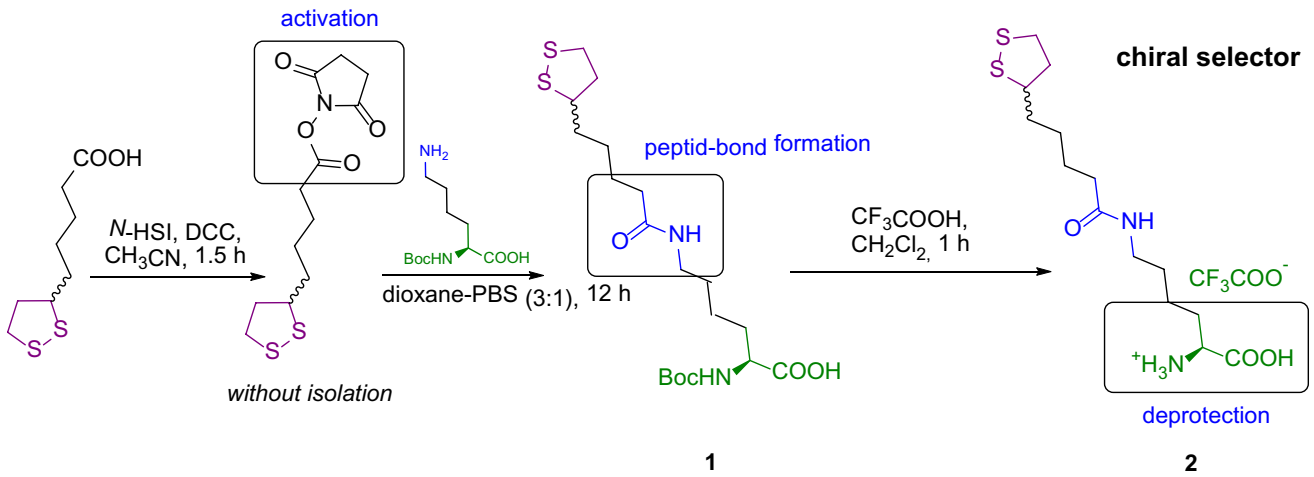


Fig. 1 Scheme of synthesis of chiral selector

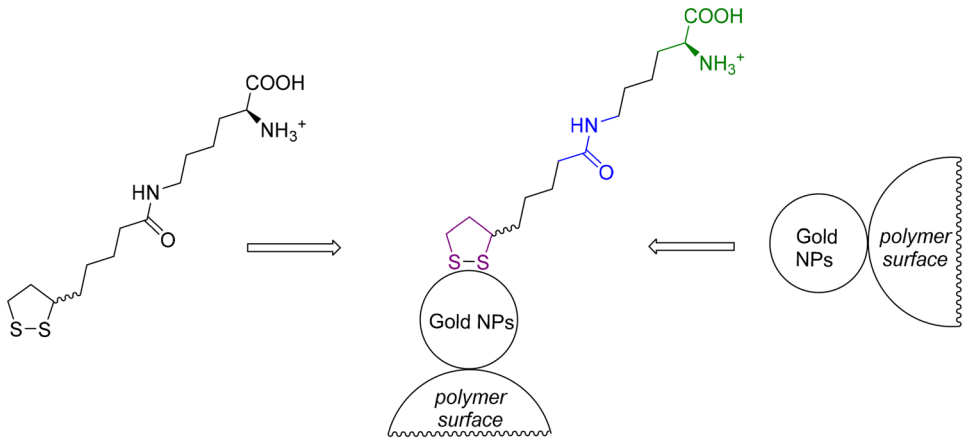


Fig. 2 Scheme of immobilization of chiral selector

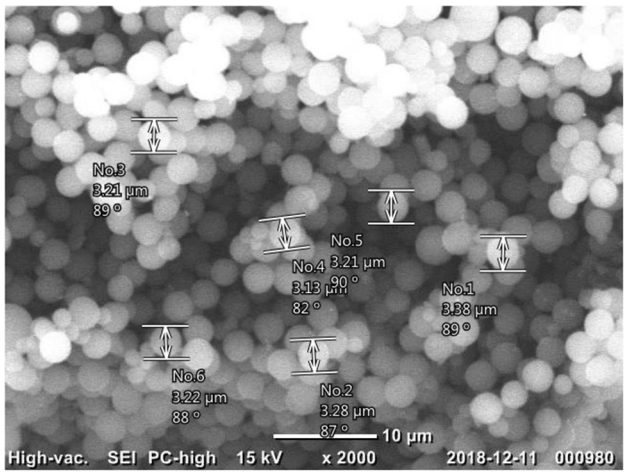


Fig. 3 SEM image of PS-DVB-Au-LA-Lys particles

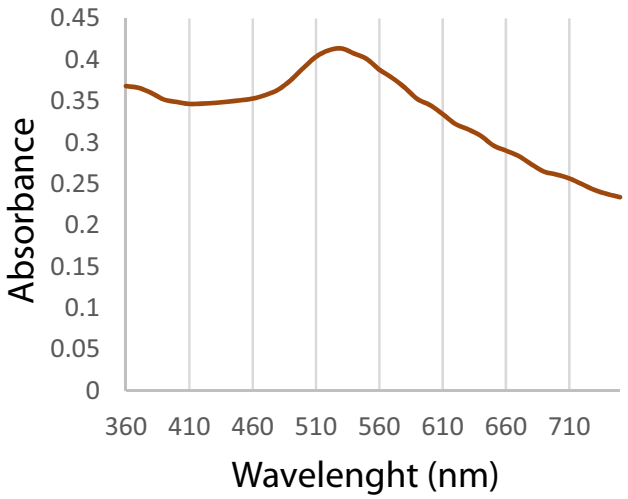


Fig. 4 Diffuse reflection spectrum of PS-DVB-Au-LA-Lys

The prepared composite CSP PS-DVB-Au-LA-Lys was characterized by different methods. The particle size distribution of resulting microspherical adsorbent was evaluated by SEM. The corresponding SEM image is shown in Fig. 3. CSP particles have a spherical shape and a diameter between 3.0 and 3.3 µm. Clearly, the modification did not affect the size and shape of original PS-DVB microspheres.

The PS-DVB-Au-LA-Lys was also characterized by using diffuse reflection spectroscopy. The obtained spectrum (Fig. 4) has a clear peak with absorption maximum at 520–540 nm confirming the presence of AuNPs on CSP surface as described earlier in the literature [21].

Porous structure of stationary phase is an important factor affecting its chromatographic performance. The original PS-DVB and obtained PS-DVB-Au-LA-Lys sorbents were characterized by BET method using data on adsorption of nitrogen at low temperatures. A static adsorption mode was used and included full equilibration after each adsorbate load. Adsorbent pore volume was calculated from the upper plateau of the adsorption isotherm, which corresponds to complete filling of pores [22]. The values of specific surface area, pore volume and pore size distribution measured for both adsorbents are shown in Table 3. Evidently, the values of specific surface area and pore volume are bigger for resulting CSP, while the pore size is smaller. This may be due to the larger surface-area/volume ratio of immobilized AuNPs that resulted in increased surface area, but filling the pores of the original PS-DVB matrix and the formation of new pores between AuNP particles decreases average pore

Table 3 Porous structure of original PS-DVB matrix and prepared CSP PS-DVB-Au-LA-Lys

Sorbent	Surface area (m^2/g)	Pore volume (cm^3/g)	Pore diameter (nm)
PS-DVB	301	0.53	6.5
PS-DVB-Au-LA-Lys	432	0.62	5.4

size. The immobilisation of AuNPs onto surface of PS-DVB results in increase of surface area of substrate and, hence, in loading capacity of the chiral stationary phase, while reduction of surface area is normally observed after immobilisation of organic molecules or polymer layers [23].

3.1 Chromatographic performance of CSP

In recent years, the growing attention has been noted to the development of novel stationary phase for HPLC, in order to decrease the analysis time, and to improve sensitivity, selectivity and separation efficiency. Nanoparticle containing composite materials represents one of the most promising classes of stationary phases for HPLC [1].

The prepared CSP was packed into 100 × 4.6 I.D. mm stainless steel column using the slurry packing procedure. The chromatographic performance of the column was checked in reversed-phase mode of HPLC. The chromatographic retention of profens and β-blockers on the obtained CSP was studied depending on the composition of the mobile phase including type of the buffer solution, its concentration, pH, organic solvent content and its nature. Under optimized conditions a complete separation of six β-blockers was obtained in 15 min (Fig. 5).

The studied β-blockers have one (atenolol, metoprolol, oxprenolol, alprenolol) or two (nadolol, pindolol) aromatic rings, secondary amino group with pKa in the range from 8.8 to 9.7, hydroxy- and ether groups in their molecules with well documented hydrophobic properties. The corresponding pKa and logP values are presented in Table 4. The prepared CSP has also complex structure with various possibilities for mixed-mode retention for β-blockers due to hydrophobic, electrostatic, hydrogen bonding and π-π interactions. It was found that retention of β-blockers on both bare PS-DVB column and column packed with prepared chiral stationary phase PS-DVB-Au-LA-Lys is well correlated with their hydrophobicity estimated by using logP values, while retention on the latter column is significantly stronger. Hydrophobic interactions of solutes can be due to interactions either with surface of PS-DVB matrix or with alkyl moieties of chiral selector immobilized onto gold nanoparticles. Obviously, the stronger retention of β-blockers on PS-DVB-Au-LA-Lys as compared with bare PS-DVB is associated with increase in surface area from 301 to 432 m^2/g due to coating with layer of gold nanoparticles modified with hydrophobic chiral selector.

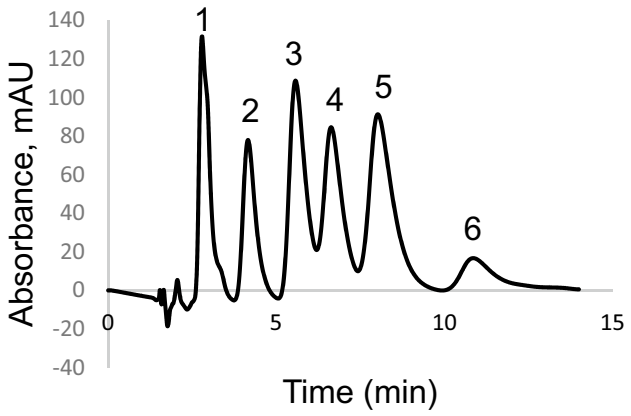


Fig. 5 Chromatogram of β-blockers: 1 atenolol, 2 nadolol, 3 pindolol, 4 metoprolol, 5 oxprenolol, 6 alprenolol. Mobile phase: ACN/MeOH/10 mM phosphate buffer (pH 6.8) (40:40:20 vol%), flow rate 0.5 mL/min, 230 nm

Table 4 pKa [24] and log P [25]) values and retention factors of selected β-blockers on columns packed with bare PS-DVB and PS-DVB with immobilised gold nanoparticles modified with chiral selector LA-Lys

Analytes	pK_a	Log *P*	Retention factor, *k′*	
			PS-DVB-Au-LA-Lys	PS-DVB
Atenolol	9.60	0.16	0.77	0.49
Nadolol	9.67	0.81	1.58	0.75
Pindolol	8.8	1.75	2.4	1.22
Metoprolol	9.7	1.88	3.06	1.91
Oxprenolol	9.5	2.10	3.89	2.25
Alprenolol	9.6	3.10	5.41	3.15

Mobile phase: ACN/MeOH/10 mM phosphate buffer (pH 6.8) (40:40:20 vol%)

The chiral recognition of profens enantiomers was found for the prepared CSP. The obtained chromatograms of flurbiprofen and ketoprofen enantiomers with peak resolution (R_S) 0.96 and 0.85, respectively, are shown in Fig. 6.

The developed CSP was used for everyday chromatographic experiments during two months without any noticeable changes in its properties.

4 Conclusions

The chiral selector **2** was synthesized and its chemical structure was confirmed. This selector was used for the preparation of new chiral stationary phase by modification of AuNPs coated PS-DVB microparticles. The advantage of PS-DVB-Au is associated with an increase in specific surface area as compared with original PS-DVB matrix. It was shown that the obtained adsorbent could be used as a stationary phase for reversed-phase HPLC and for the separation of profens enantiomers.

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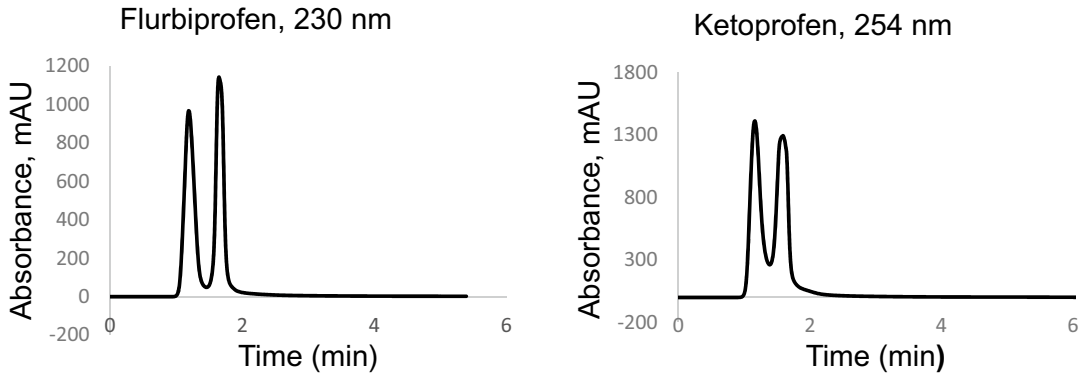

Fig. 6 Chromatograms of profens enantiomers. Mobile phase: ACN/0.05% TEAA (pH 7) (90:10 vol%), flow rate 0.5 mL/min

Author contributions Conceptualization: IAA, PNN; methodology: EKB, AYP, DSP; formal analysis and Investigation: DSP, AYP; writing—original draft preparation: DSP, AYP; writing—review & editing: PNN, EKB, IAA; funding acquisition: IAA; supervision: IAA. All authors read and approved the final manuscript.

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Data Availability The data that support the findings of this study are available from the corresponding author Prosuntsova D.S., upon reasonable request.Code availability Not applicable as all data can be requested from the corresponding author Prosuntsova D.S., upon reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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