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MICROSPHERICAL POLYSTYRENE-DIVINYLBENZENE PARTICLES HYBRIDIZED WITH EREMOMYCIN STABILIZED GOLD NANOPARTICLES AS A STATIONARY PHASE FOR CHIRAL LIQUID CHROMATOGRAPHY

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A novel enantioselective adsorbent was obtained by hybridization of microspherical polystyrene-divinylbenzene (PS-DVB) macroporous particles with eremomycin-stabilized gold nanoparticles (GNPs). Macrocyclic antibiotic eremomycin was used as a stabilization agent to obtain GNPs which were then characterized by transmission electron microscope. The average diameter of obtained nanoparticles is about 16.6 nm. Eremomycin-stabilized nanoparticles were successfully embedded into the porous polymer structure with a resulting chiral selector content of 37.5 μmol/g. The obtained PS-DVB composite containing GNPs with immobilized eremomycin was studied by scanning electron microscopy and diffuse reflectance spectroscopy. The values of the specific surface area (500 m^2/g) and porosity of the adsorbent (0.39 cm^3/g) are measured using nitrogen adsorption at low temperatures. The obtained composite material was used as a chiral stationary phase of liquid chromatography. A good separation enantioselectivity to amino acids, their derivatives and beta-blockers under RPC (reversed-phase) and HILIC (Hidrophilic Interaction Liquid Chromatography) mode is demonstrated. The results obtained revealed that the prepared Eremo@GNP@PS-DVB composite is promising for use as a stationary phase in HPLC.

Keywords: poly(styrene-divinylbenzene); gold nanoparticles; liquid chromatography; chiral stationary phase; macrocyclic antibiotic.

Introduction

Composite polymeric materials have recently been actively studied and found their application in various fields. One of the most popular composite stationary phases for high-performance liquid chromatography are agglomerated or nanoparticle coated materials [1]. Nanoparticles (NPs) have been successfully used in separation science in the past decade due to their unique properties that included a large surface area-to-volume ratio, simplicity of coating and unique physico-chemical properties [2].

So far, various types of functional inorganic nanoparticles have been assembled on the surfaces of carbonaceous and polymeric microspheres, resulting in organic/inorganic composite particles. Wiest et al. showed the advancing of the microspherical particles with carbon cores and detonation nanodiamond (DND) containing shells as stationary phase in high performance liquid chromatography (HPLC) [3, 4]. The addition of DND increased the mechanical stability and thermal conductivity of the prepared core-shell stationary phase. The properties and applications of HPLC stationary phases containing other types of carbon nanoparticles (carbon nanotubes, nanographen, carbon dots) [5], metal oxides (titania, alumina, zirconia, iron oxides) [6], hydroxyapatite [7], latexes [8], and metal [9] nanoparticles have been also reviewed.

For the latter type of nanoparticles used for the preparation of HPLC stationary phases a great attention was paid to the application of noble metals nanoparticles, especially gold nanoparticles (GNP). This is due to high affinity of noble metal nanoparticles (Au, Ag, Pt, and Pd) to porous polymer matrices, which simplifies the preparation of corresponding adsorbents. The hybrid structure with polymers allows the noble metal nanoparticles to retain high stability against aggregation. Li et al. developed a facile and controllable strategy for the preparation of composite particles composed of polystyrene (PS) microspheres decorated with GNPs through Pickering emulsion template [10]. The method enables easy and effective control over

the size and surface coverage of PS microspheres with nanoparticles. The GNP@PS particles have been used by Kobayashi et al. to prepare a stationary phase for capillary liquid chromatography [11]. Importantly, the surface of GNPs was additionally modified with thiol compounds (n-octadecanethiol, thiophenol, and 2-phenylethanethiol) through the formation of self-assembled monolayer. The resulting thiol@GNP@PS stationary phases revealed their significantly unique retention behaviors as compared to the conventional ODS column.

The use of nanomaterials for chiral separation is a current trend in the development of chiral stationary phases (CSP) for HPLC [12]. Obviously, this type CSPs can be prepared by adsorption modification of GNP surface with appropriate chiral selectors. In our previous work HPLC chiral stationary phase was prepared by coating of poly(styrene-divinylbenzene) (PS-DVB) microspherical particles with GNPs followed by modification of gold surface with L-lysine conjugated with lipoic acid [13]. The prepared CSP demonstrated a good resolution of profens enantiomers. However, the search for more selective and versatile ligands for the preparation of this type CSP is a current topic.

Due to presence of multiple chiral centers macrocyclic antibiotics are widely used as universal chiral selectors in HPLC [14, 15]. Among them, the glycopeptide macrocyclic antibiotic eremomycin exhibited a high enantioselectivity. The chemical structure of eremomycin molecule includes 22 chiral centers, 3 cavities, 3 sugar moieties, 5 aromatic rings, 1 carboxylic group, 9 hydroxyl groups, 7 amido groups and 3 amino groups [16]. These structural elements can provide multiple combinations of hydrophobic inclusion, hydrogen bonding, dipole-dipole, $\pi - \pi$ interactions, as well as steric hindrance effects influencing chiral recognition and separation enantioselectivity. Eremomycin bonded silica CSP has been successfully applied to HPLC separation of α-amino acids [16 – 18], α-hydroxy acids and their derivatives [19, 20], α-methylphenylcarboxylic acids (profens) [21 – 24] and dipeptides [25] stereoisomers.

However, the applicability of eremomicyn complexes with GNPs remain vastly unexplored. There is only one communication on using silica based CSP modified with eremomycin-stabilized GNPs for the separation of aromatic amino acids [26]. The present investigation deals with preparation and characterization of new CSP composed of porous polymer core containing a layer of eremomycin stabilized GNPs. To evaluate possible applications of this new sorbent, its hydrophilic, acid-base and enantioselective properties were investigated.

Experimental

Materials and methods. To prepare a new adsorbent $HAuCl_4 \cdot 3H_2O$ (Au-%, 50, ChemPur), eremomycin hydrochloride ("BioKhimMak ST," Moscow, Russia) and disodium tetraborate are used. PS-DVB microspherical particles (size 5 μm, 20 – 30 nm pore size and 80 % crosslinking degree) obtained from "Technosorbent" (Moscow, Russia) are used as a matrix.

The following reagents are used as model analytes: uridine, 5-methyluridine, 2-deoxyuridine, theobromine, theophylline, toluene, uracil, alanine, phenylalanine, benzoyl-phenylalanine, CBZ-asparagine, CBZ-tryptophan, BOC-alanine, BOC-valine, atenolol, pindolol, metoprolol, oxprenolol, alprenolol, nadolol (Sigma-Aldrich). HPLC-grade methanol ("J. T. Baker," Netherlands), acetonitrile ("Panreac," Spain) and Milli Q deionised water (Millipore, Milford, MA, USA) were used for preparation of all eluents and sample mixtures.

Synthesis of the hybrid stationary phase. $HAuCl_4 \cdot 3H_2O$ (33 mg) was placed in a 100 mL round-bottom flask, then 24 mL of a 0.05 M borate buffer solution was added and stirred until complete dissolution of tetrachloroauric acid. Eremomycin hydrochloride (0.88 g) was diluted in 40 mL of borate buffer and mixed with gold solution. An additional 16 mL of borate buffer was added and mixed. The solution was heated in a round bottom flask under reflux for 1.5 – 2 h until appearance of a dark ruby color product. 3 grams of PS-DVB microparticles were added to 100 mL of aqueous solution of eremomycin-stabilized GNPs and obtained suspension was stirred at room temperature. After 5 h, prepared Eremo@Au@PS-DVB particles were filtered using a 0.22 μm nylon membrane and washed with water and methanol.

Instruments. The HPLC experiments were performed on a Shimadzu LC-20 Prominence liquid chromatograph (Japan) consisting of a solvent delivery pump (LC-20AB), degasser (DGU-20A5), column oven (CTO-20A) and a diode array detector (SPD-M20A). Data collection and the processing of chromatograms were carried out using the Shimadzu LC Solution software. pH measurements were carried out using a pH meter "Starter 2100" ("OHAUS," Russia). All solvents were degassed by an ultrasonic bath ("Sapphire 6580," Russia).

The Eremo@GNP@PS-DVB particles as stationary phase for HPLC were slurry packed into the stainless steel tube (100 × 4.6 mm) through pressing with methanol/deionized water (50/50) as packing solvent under a working pressure of 40 MPa. The volume of packing solvent passing through the column should be at least 500 mL. Approximately 1.4 g of the stationary phase was used to pack the column.

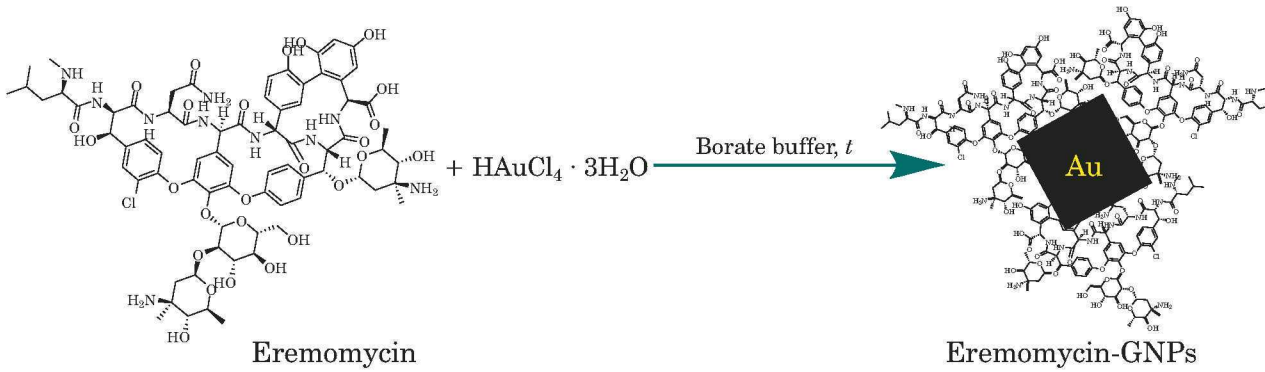

Fig. 1. Schematic illustration of the synthesis of Eremo@GNPs

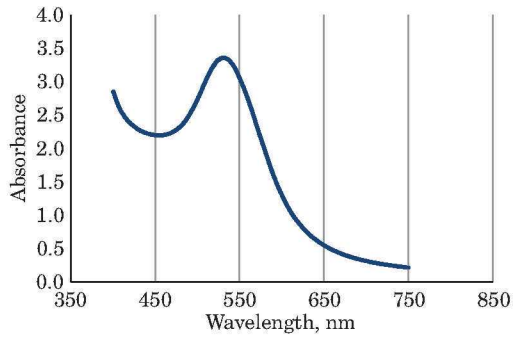

Fig. 2. UV-Vis spectrum of Eremo@GNPs exhibited surface plasmon band with maximum at 530 nm

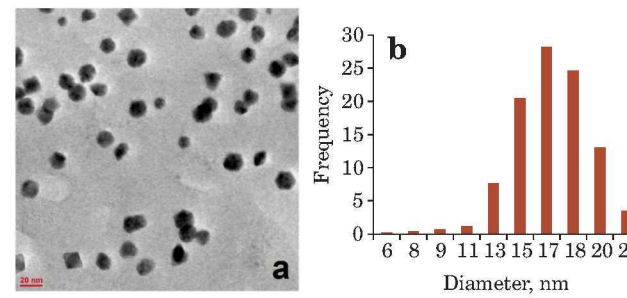

Fig. 3. TEM images (*a*) and particle size distribution (*b*) of Eremo@GNPs

Morphological and physicochemical characterizations. Gold nanoparticles were characterized with a JEOL JEM-2100 F/Cs/GIF/EDS transmission electron microscope (TEM) (Jeol, Japan).

The size and shape of gold-coated polymer particles were determined using a JEOL JCM-6000 scanning electron microscope (SEM) (Tokyo, Japan) at 20 kV.

The dried sorbent was pressed into tablets and the diffuse reflection spectra were recorded on a CS-9001PC Dual-Wavelength Flying Spot Scanner (Shimadzu, Japan).

Spectrophotometric studies were carried out on a U-2900 double beam spectrophotometer (Hitachi, Japan).

The surface area and the pore-size were determined by N_2 adsorption-desorption at 77 K, using Brunauer – Emmett – Teller (BET) and Barrett – Joyner – Halenda (BJH) methods, respectively. Measurements were made by a Micromeritics instrument, Model ASAP 2020 (Norcross, USA), for samples previously desorbed for 24 h at 100°C under a vacuum before recording their isotherms.

Approximately 10 mg of each sample was subjected to elemental analysis on a Model 2400 Series II CHNS/O Perkin Elmer Analyzer (Shelton, USA).

The concentration of Au was measured by microwave coupled plasma atomic emission spectrometry (AES) (Agilent 4200 MP-AES, USA) using the standard samples in 0.1 mg/mL concentration range for calibration.

Results and Discussion

Synthesis and characterization of Eremo@GNPs. Nitrogen-containing antibiotics can be used for a single-step obtain Eremo@GNPs directly by the reduction of tetrachloroauric acid with eremomycin solution avoiding an intermediate step in the synthesis of citrate-stabilized GNPs. For this purpose a mixture of $HAuCl_4 \cdot 3H_2O$ and eremomycin in a borate buffer solution was heated in a round bottom flask under reflux until an appearance of rich ruby color according to procedure described by Shapovalova et al. [26] (Fig. 1).

The UV-visible spectra of Eremo@GNPs solution has a peak with maximum around 530 nm (Fig. 2), which is in agreement with the characteristic plasmonic absorption bands of GNPs [27]. To investigate the morphology and particle size, TEM was used (Fig. 3). According to the obtained image Eremo@GNPs have mostly spherical shape of 13 – 20 nm diameter, while the average diameter is equal to 16.6 nm.

Preparation of Eremo@GNP@PS-DVB particles. The prepared Eremo@GNPs were immobilized onto the surface of PS-DVB spherical microparticles by using physical adsorption. The resul-

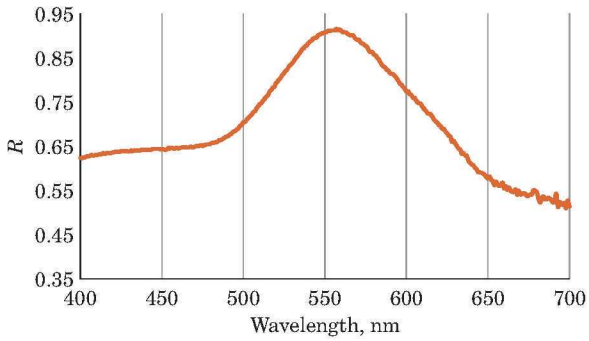

Fig. 4. The diffuse reflection spectrum of Eremo@GNP@PS-DVB composite

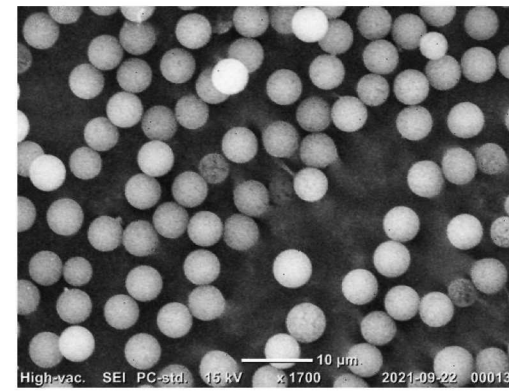

Fig. 5. SEM images for Eremo@GNP@PS-DVB particles

tant Eremo@GNP@PS-DVB composite has typical raspberry-like color. To confirm the modification of polymer matrix with the gold nanoparticles, diffuse reflectance spectroscopy (DRS) was used. The absorption maximum in the region of 550 nm in the DRS spectrum proves the presence of GNPs in a non-aggregated state on the sorbent surface (Fig. 4). The Au content was 1.39 % wt. according to the AES analysis. The mass of a single obtained GNP is equal to 4.5×10^{-17} g by assuming average particle diameter 16.6 nm, determined earlier, and metal gold density 19.0 g/cm^3. Hence, the resulting concentration of GNPs immobilized onto PS-DVB matrix is approximately equal to 3.06×10^{14} particles per gram. The amount of eremomycin, calculated from the nitrogen content, obtained by elemental analysis, was 37.5 µmol/g, which is two times higher than that previously reported for a silica gel modified with Eremo@GNPs [26].

The surface properties of a chromatographic support played an important role in the separation performance and selectivity, and also in the retention parameters of liquid chromatography. The particles of the prepared composite were examined by SEM. SEM images showed no visible aggregation and destruction of Eremo@GNP@PS-DVB particles (Fig. 5). The uniform-sized spherical shape particles indicated that the sorbent have not undergone any significant change in shape during eremomycin-stabilized GNPs loading.

The porous structure of the prepared adsorbent, as well as unmodified PS-DVB, was characterized by using low-temperature nitrogen adsorption method. The specific surface area has been calculated by BET method and porosity by BJH method. The obtained results are shown in Table 1.

The reduction of the specific surface area from 610 to 500 m^2/g and pore volume from 0.47 to 0.39 cm^3/g indicates the filling pores with Eremo@GNP particles. At the same time, some increase in micropore volume for the modified PS-DVB as compared with bare PS-DVB is noted. Obviously, this is connected with the formation of new micropores formed by nanoparticles and the rest of the pore volume that also indicate the deposition of Eremo@GNPs into polymer macropores. This was expected, since the pore size of the initial polymer is 20 – 30 nm, and the diameter of the obtained nanoparticles does not exceed 20 nm. The results showed that the surface characteristics of Eremo@GNP@PS-DVB complied with the requirements for materials to be used in LC.

Chromatographic investigation of Eremo@GNP@PS-DVB stationary phase. The adsorption properties and chromatographic performance of both PS-DVB and Eremo@GNP@PS-DVB microspheres were evaluated under HPLC mode with 100 × 4.6 mm I.D. stainless steel column slurry packed with corresponding adsorbents. Hydrophilic and hydrophobic interactions make a significant contribution to the retention of analytes in reverse-phase chromatography and Hydrophilic Interaction Liquid Chromatography (HILIC). To estimate hydrophobic properties of the sorbents, the methylene selectivity values $\alpha(CH_2)$ are normaly used according to Tanaka test [28] designed for the characterization of stationary phases. In HILIC the value of $\alpha(CH_2)$ is expressed as ratio of retention factors of uridine and 5-methyluridine k(uridine)/k(5-methyluridine). Since 5-methyluridine has an extra methyl group, which contributes to

Table 1. Surface characteristics of original PS-DVB matrix and Eremo@GNP@PS-DVB

Sorbent	Surface area, m^2/g	Pore volume, cm^3/g	Micropore volume, cm^3/g
PS-DVB	610	0.47	0.04
Eremo@GNP@PS-DVB	500	0.39	0.06

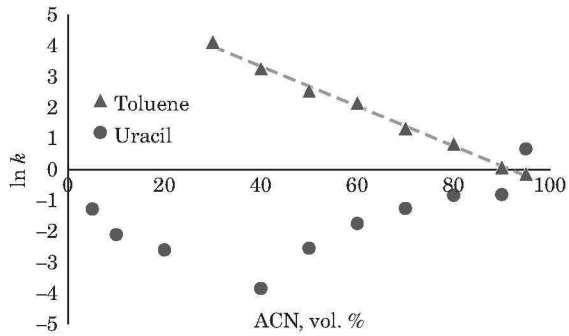

Fig. 6. Dependence of $\ln k$ of uracil and toluene on the acetonitrile concentration in the eluent (column: Eremo@GNP@PS-DVB, 100 × 4.6 mm ID; eluent: acetonitrile — 10 mM ammonium acetate buffer with pH 5.2; flow rate 1 mL/min)

stronger hydrophobic interactions, which resulted in a weaker retention under HILIC conditions. Similarly, the hydrophilic interactions are evaluated using a selectivity towards hydroxyl group α(OH) by comparing the retention factors of uridine and 2'-deoxyuridine, which lacks one hydroxyl group and therefore it is less hydrophilic than uridine. The greater selectivity factor α(OH) means the more pronounced hydrophilic properties of the stationary phase. The acid-base nature of the stationary phase also has a significant effect on the separation of substances, especially those containing ionic groups and participating in electrostatic interactions. The substances theophylline (Tp) and theobromine (Tb) having pK_a values 8.6 and 10, respectively, so theobromine is stronger retained by acidic adsorbents than theophylline. The value of the parameter α(OH) (Table 2) is higher than of parameter $\alpha(CH_2)$, which indicate that Eremo@GNP@PS-DVB stationary phase reveals the hydrophilic properties. The ratio of retention factors k(Tb)/k(Tp) higher than 1 means that the stationary phase is rather acidic [28].

The retention factors of uridine and toluene were compared for unmodified PS-DVB column and Eremo@GNP@PS-DVB, the obtained results are presented in Table 2. The toluene having no hydrophilic functional groups in the molecule is retained stronger than uridine, which indicates the presence of some hydrophobic interactions for the prepared CSP. However, the retention decreases with increase of organic solvent content, while the retention of uridine increases too. It indicates the strengthening of hydrophilic interactions contribution in the retention of model compounds for the polymer after modification with eremomycin, which contains many of polar groups in the molecule.

The effect organic solvent concentration in the eluent was investigated more carefully for toluene and uracil used as test probes for hydrophobic and hydrophilic interactions, respectively (Fig. 6). Depending on concentration of acetonitrile in the mobile phase Eremo@GNP@PS-DVB material can exhibit either reverse-phase or HILIC-mode retention characteristics. As illustrated in Fig. 6, at acetonitrile concentrations from 40 to 95 vol.%, the retention of uracil increased with growing concentration of acetonitrile that is in agreement with HILIC behavior of CSP. At concentration from 5 to 40% acetonitrile the retention of uracil decreased with increase in the concentration of acetonitrile, which is typical for a dominance of reversed-phase retention mechanism. The retention of toluene is stronger and it is decreased with increase in the acetonitrile content in the mobile phase for the whole range of studied concentrations. The dependence of $\ln k$ of toluene on acetonitrile content is approximately linear, which indicates a purely reversed-phase mechanism associated with hydrophobic and $\pi - \pi$ interactions between aromatic rings of analytes and PS-DVB matrix.

Enantioselective properties of CSP. Eremomycin has several chiral centers, so, potentially, the prepared composite adsorbent can separate enantiomers. This possibility was investigated for various model compounds under reversed-phase conditions. The mixture of methanol and acetonitrile at various concentrations in the eluent and different pH buffers are investigated. The prepared composite CSP showed good enantioselectivity for amino acids, their derivatives and beta-blockers pindolol and nadolol. The corresponding values of resolutions (Rs) and separation factors (α) for selected enantiomers are given in Table 3. The example of enantiomers separation is shown for CBZ-tryptophan in Fig. 7.

The baseline separation for mixture of four different beta-blockers, common pharmaceutical compounds, can be also achieved on Eremo@GNP@PS-DVB column with alkaline mobile phase at pH 10 (Table 4). This is possible due to the high hydrolytic stability of the developed stationary

Table 2. Separation factors for methylene $\alpha(CH_2)$ and hydroxy α(OH) groups and retention factors for uridine and toluene (eluent: acetonitrile — 20 mM ammonium acetate, pH 4.7 (90:10, v/v); flow rate 0.5 mL/min)

Sorbent	k(uridine)	k(toluene)	$\alpha(CH_2)$	α(OH)	α(k(Tb)/k(Tp))
Eremo@GNP@PS-DVB	0.6	1.05	1.5	3.0	1.5
PS-DVB	0.1	1.75	1.0	1.0	0.9

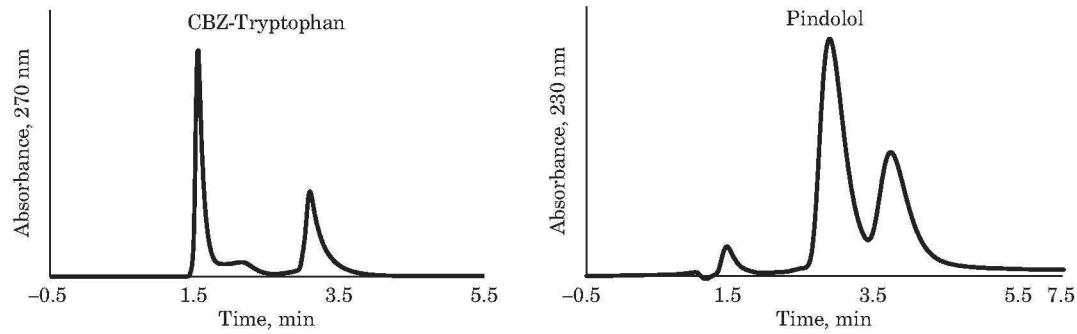

Fig. 7. The enantiomers separation of CBZ-tryptophan and pindolol (flow rate 0.5 mL/min; see conditions in Tables 3 and 4)

Table 3. Resolutions (*Rs*) and separation (α) factors of selected compounds for Eremo@GNP@PS-DVB column

Analyte	Mobile Phase	*Rs*	α
Alanine	MeOH: 0.1 M NH_4OAc, pH 5.6 (60:40, v/v)	1.9	2.8
Benzoyl-phenylalanine		0.9	1.4
CBZ-asparagine	MeOH:0.1 M NH_4OAc, pH 5.6 (90:10, v/v)	1.2	5.1
Phenylalanine	ACN:MeOH:TEA:HOAc (84.9:15:0.05:0.05, v/v/v/v)	1.2	4.1
CBZ-tryptophan	ACN:TEA:HOAc (99.8:0.1:0.1, v/v/v)	4.4	12
BOC-alanine	MeOH:TEA:HOAc (99.8:0.1:0, 1, v/v/v)	0.7	3.0
BOC-valine		0.8	3.4
Pindolol	MeOH:TEA:HOAc (99.98:0.01:0.01, v/v/v)	1.0	1.5
Nadolol	MeOH:NH_4OAc (0.1 M, pH 5.6) (98:2, v/v)	0.9	1.4

phase in highly alkaline eluent, which are required for the deprotonation of amino groups in β-blockers. Table 3 shows that separation of enantiomers for several beta-blockers is also possible. As example the separation of pindolol enantiomers is presented in Fig. 7.

CONCLUSIONS

A new composite adsorbent composed of PS-DVB matrix with physically adsorbed eremomycin-stabilized GNPs is prepared and characterized. By using SEM and atomic emission spectrometry methods it is demonstrated that Eremo@GNP particles are successfully entrapped into the porous structure of polymer microspheres by a simple adsorption. The obtained results showed that the modification of PS-DVB with eremomycin-stabilized GNPs does not produce any aggregation or shape change of the support particles. The possibility of application of the prepared adsorbent as a stationary phase for chiral liquid chromatography is demonstrated. The obtained chiral stationary phase demonstrated the ability to work both in reversed-phase and HILIC modes of liquid chromatography and under strong alkaline conditions. Eremo@GNP@PS-DVB column was used for enantiomeric separation of amino acids, their derivatives and beta-blockers.

Table 4. pK_a, retention (*k*) and separation (α) factors of β-blockers (eluent: acetonitrile — 20 mM ammonium acetate, pH 10 (80:20, v/v); flow rate 0.5 ml/min, 230 nm)

β-Blockers	pK_a	*k*	α
Atenolol	9.6	1.05	3.13
Pindolol	8.8	3.27	
			1.53
Oxprenolol	9.5	5.00	
			1.45
Alprenolol	9.6	7.24	

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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