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Binding of human serum albumin to silica particles by means of polymers: a liquid chromatographic study of the selectivity of resulting chiral stationary phases

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Abstract

Chiral stationary phases obtained by immobilization of human serum albumin (HSA) on various polymer-coated silicas were tested to resolve DL-tryptophan, DL-NBP, *RS*-oxazepam and *RS*-warfarin racemic mixtures. HSA immobilized on anion exchangers [quaternized poly(vinylimidazole)-coated silica] was highly selective. Stable and selective chiral stationary phases were also prepared by covalent binding of HSA to silica particles via reactive-polymers. Poly(acryloyl chloride), poly(methacryloyl chloride) and poly(vinyl chloroformate) derivatives were compared. Parameters that govern the selectivity of resulting chiral supports were evaluated, especially the orientation of HSA after immobilization, the mobility of polymer chains and the number of covalent linkages between the protein and the polymer.

Keywords: Chiral stationary phases, LC; Human serum albumin; Silica; Polymers

1. Introduction

Proteins immobilized on solid supports are widely used for biological applications such as enzymatic reactions [1,2], purifications by immunoaffinity chromatography [3,4], and chiral separations [5–9]. However, immobilization procedures must preserve their biological activity. The same problem is encountered in sensing technology where several types of biosensors involving biomolecules bound to flat surfaces or optical fibers have been described [10]. For this reason, methods leading to statistically negligible conformational changes are used to prepare protein-coated supports.

In most cases, matrices employed to immobilize proteins are hydrophilic to avoid hydrophobic interactions between biomolecules and supports. These interactions are known to induce conformational changes which can lead to significant alteration in the biological properties of protein molecules. For example, it was shown by Fourier transform infrared spectroscopy (FT-IR) that the secondary structure of human albumin was extensively modified after adsorption on reversed-phase supports [11,12]. Moreover, conformational changes were also observed in hydrophobic interaction chromatography [13] in spite of the weak hydrophobicity of supports used in this technique, affecting retention and peak shape of proteins. It was shown that structural changes in-

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creased with the hydrophobic character of the stationary phase and depended on the lability of the protein.

In this paper, immobilization procedures using an intermediate polymer layer to bind human serum albumin (HSA) to silica particles are reported. Parameters that govern the enantioselective properties of the resulting materials are explored by high-performance liquid chromatography (HPLC).

2. Experimental

2.1. Reagents

Porous LiChrospher Si300 (d_p=10 μm; porosity 300 Å) and cysteamine hydrochloride were purchased from Merck (Darmstadt, Germany). Nucleosil-NH_2 (d_p=7 μm; porosity 300 Å) was from Macherey-Nagel (Düren, Germany). *N*-vinylimidazole (VI), acryloyl chloride, methacryloyl chloride, vinyl chloroformate, azobis(isobutyronitrile) (AIBN), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), 2,2′-dipyridyl disulfide (2-PDS), 2-thiopyridone (2-TP), 1,4-butanediol diglycidyl ether (BUDGE), *N*-hydroxysuccinimide (NHS), ethanolamine and *RS*-warfarin were from Aldrich (Steinheim, Germany). Human serum albumin, DL-tryptophan, *N*-benzoyl-DL-phenylalanine (DL-NBP) were purchased from Sigma (St. Louis, MO, USA). *RS*-Oxazepam was obtained from Sanofi (Toulouse, France). Solvents and buffer constituents were of analytical-reagent grade.

2.2. Preparation and quaternization of PVI coated silicas: [PVI$^+$-Sil]

Poly(vinylimidazole) (PVI; number-average molecular mass: 40 000 g/mol) was prepared and adsorbed on LiChrospher Si300 as described previously [14]. Crosslinking and quaternization of the coated polymer layer were performed with BUDGE (6 mmol per g of silica) at 60°C, in ethanol (2 h).

2.3. Coupling of HSA to [PVI$^+$-Sil]

[PVI$^+$-Sil] was slurry packed in a stainless steel column (100×4.6 mm). The HSA solution (2 g/l) in 0.067 *M* phosphate buffer, pH 7.4, containing various concentrations of sodium chloride was applied to the column until saturation. Thereafter, columns were washed with 0.067 *M* phosphate buffer. The amounts of HSA bound to the columns were determined from the HPLC analysis of the fractions collected at the column outlet during the percolation of HSA and the washing steps.

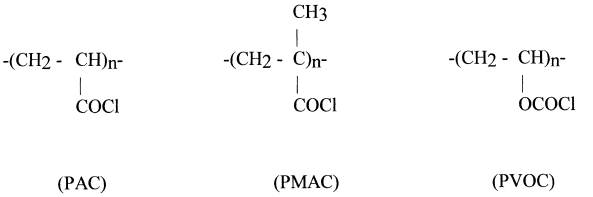
2.4. Preparation of PAC and PMAC coated silicas: [PAC-Sil] and [PMAC-Sil]

As described previously [15,16], poly(acryloyl chloride) (PAC) and poly(methacryloyl chloride) (PMAC; Scheme 1) were synthesized by radical polymerization in 1,2-dichloroethane and 1,4-dioxane, respectively. The respective number-average molecular masses of PAC and PMAC were 40 000 and 75 000 g/mol, respectively.

Then amino-silica (Nucleosil-NH_2) dried overnight at 50°C was reacted during 5 h with a 5% (w/w) solution of reactive PAC or PMAC, either in dry 1,2-dichloroethane or dry 1,4-dioxane and washed extensively.

2.5. Coupling of HSA to [PAC-Sil] and [PMAC-Sil] supports

The acyl chloride functions of the immobilized polymer were hydrolyzed at 45°C in a pH 7.8 phosphate buffer (0.15 *M*). The resulting carboxylic groups were reacted with NHS in the presence of EDC to give NHS-ester functions able to react quickly with NH_2 groups of the protein [15,16]. [PAC-NHS] and [PMAC-NHS] stationary phases were slurry packed in 150×4.6 mm columns. Then the HSA solution (2 g/l) in phosphate buffer (50 m*M*, pH 7.4, with 0.5 *M* NaCl) was applied to the column until equilibrium. The amounts of HSA



Scheme 1.

bound to the columns were determined as described for [PVI$^+$-HSA] supports. After coupling of HSA, unreacted NHS-ester groups were blocked with ethanolamine (0.5 *M*, pH 7.8).

2.6. Preparation of PVOC coated silicas: [PVOC-Sil]

Poly(vinyl chloroformate) (PVOC; Scheme 1) was synthesized by radical polymerization of freshly distilled vinyl chloroformate (10 ml) in 1,2-dichloroethane (85 ml), in the presence of 0.35 g of dicyclohexylperoxydicarbonate (DCPD). Polymerization was carried out at 60°C, for 5 h, in a nitrogen atmosphere. The polymer was purified by precipitation in diethyl ether. The number-average molecular mass of the PVOC polymer was 52 000 g/mol.

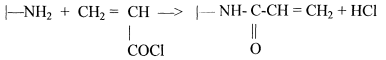
Then amino-silica (Nucleosil NH_2) dried overnight at 50°C was reacted (5 h) with a 5% (w/w) solution of reactive PVOC in dry 1,2-dichloroethane (10 ml per g of amino-silica) and washed extensively with 1,2-dichloroethane.

2.7. Coupling of HSA to [PVOC-Sil] supports

PVOC-coated silica was reacted 5 h with a 0.3 *M* solution of NHS in dry acetonitrile (10 ml per gram of silica) to give NHS-carbonate functions. After rinsing with acetonitrile, [PVOC-NHS] silica was slurry packed in a 150×4.6 mm column. Then the protein solution (2 g/l) in phosphate buffer (50 m*M*, pH 7.4, with 0.5 *M* NaCl) was applied to the column until equilibrium and the amount of HSA bound to the column determined. In some cases, ethanolamine (0 to 0.12 *M*) was added to the buffer during the binding of HSA to [PVOC-NHS] silica. This compound acts as a competitor.

2.8. In situ polymerization of monomers on amino silica and coupling of HSA

Amino silica (1 g) dried overnight at 50°C was reacted (Reaction 1)



Reaction 1

for 30 min at room temperature in a nitrogen atmosphere with a freshly distillated monomer solution (120 mmol in 10 ml of 1,2-dichloroethane). Then, 1.2 mmol of initiator (AIBN for acryloyl and methacryloyl chloride, DCPD for vinyl chloroformate) was added to the suspension and the mixture heated at 55°C (1 h for acryloyl chloride, 5 h for vinyl chloroformate, 18 h for methacryloyl chloride). After extensive washing, the resulting materials were reacted with NHS, as described before and packed in the columns (150×4.6 mm). Then HSA was immobilized on supports leading, respectively, to [T-PAC-HSA] and [T-PMAC-HSA] and [T-PVOC-HSA] columns.

2.9. Modification of [PVOC-Sil] by thiol functions

[PVOC-Sil] (1 g) was reacted overnight at room temperature with 20 ml of an aqueous 1 *M* solution of cysteamine hydrochloride at pH 7.4 (Reaction 2)

Reaction 2

After extensive washing, the thiol content of the resulting [PVOC-SH] support was determined with a 0.005 *M* solution of 2-PDS (Scheme 1, Reaction 3) prepared in an ethanolic phosphate buffer [pH 7; water–ethanol (50:50, v/v) mixture]. The thiol content was determined from the amount of 2-thiopyridone released by the thiol–disulfide interchange reaction (measured at 350 nm). It was equal to 10^{-4} SH per gram.

2.10. Binding of HSA to [PVOC-SH]

[PVOC-SH] silica was slurry packed in a 150× 4.6 mm column. Then a 2-PDS solution (0.005 *M*) in an ethanolic phosphate buffer (pH 7) was passed through the column until obtention of a stable baseline at 350 nm (Reaction 3)

Reaction 3

Then HSA (3 g/l in a 0.15 *M* phosphate buffer with 1 *M* NaCl and 10^{-3} *M* EDTA, pH 8) was bound to

[PVOC-SSPyr] as described previously for PVI-coated silicas bearing disulfide groups [17].

2.11. Equipment

The liquid chromatography system comprised two pumps (Waters, Milford, MA, USA, Model 501 and Spectraphysics, San Jose, CA, USA, Model SP 8810), a six-port valve (Rheodyne, Berkeley, CA, USA, Model 7010) to commute from one eluent to the other, a sample injector (Rheodyne, Berkeley, CA, USA, Model 7125), a UV variable-wavelength absorption detector (Kratos Analytical, Ramsey, NJ, USA, Model 757) and a Kipp and Zonen recorder (type BD 41).

2.12. Enantiomeric separations

All enantiomeric separations were performed under isocratic conditions at a flow-rate of 1 ml/min. The mobile phase used for the elution of racemic mixtures (DL-tryptophan, DL-NBP, *RS*-warfarin and *RS*-oxazepam) was a 50 m*M* phosphate buffer at pH 7.4. In some cases, 1-propanol was added to the mobile phase. The amounts of sample injected on the columns was equal to 0.4 μg. The wavelength used for the detection of enantiomers was 280 nm except for DL-NBP (250 nm).

Selectivity values α were calculated from retention factors k_1 and k_2 ($\alpha = k_2/k_1$).

3. Results and discussion

Proteins are known to bind to inorganic surfaces (silica, titanium oxide, gold, etc.) by physical adsorption. However, the resulting materials are not commonly used because of their instability and the time dependent conformational changes of the biological layer. To circumvent these problems, intermediate polymer layers were employed in this study to bind proteins to silica particles and avoid non-specific interactions with the matrix. Results obtained with ionic polymers and reactive polymers are summarized.

3.1. Influence of the immobilization mode on the enantioselectivity of HSA

3.1.1. Adsorption of HSA on silica modified by a polycationic polymer

We developed quaternized poly(vinylimidazole)-coated silica supports [PVI^+-silica] to immobilize HSA (isoelectric point, pI = 4.8) by electrostatic interactions [14]. The hydrophilic PVI polymer was first adsorbed on silica particles, then crosslinked and simultaneously quaternized with BUDGE. The amounts of protein bound to the anion exchanger depended on salt concentration during the immobilization of HSA (Table 1). The resulting stationary phases ([PVI^+-HSA]) were investigated for the chromatographic separation of racemic mixtures (DL-tryptophan, *N*-benzoyl-DL-phenylalanine, *RS*-oxazepam, *RS*-warfarin). It was shown they allowed good separations of D- and L-NBP (α between 1.8 and 2). Moreover, they were highly selective for the separation of DL-tryptophan and *RS*-oxazepam. A representative chromatogram for the separation of D- and L-tryptophan is depicted in Fig. 1. Some retention and selectivity values measured for the separation of DL-tryptophan and *RS*-oxazepam are reported in Table 1. It appears that good separations can be achieved in a short time (less than 3 min for tryptophan; less than 17 min for oxazepam at 1 ml/min). The excellent performance of these materials was in good agreement with results obtained on chiral stationary phases (CSPs) described by Jacobson and Guiochon who used bovine serum albumin

Table 1
Maximum amounts of HSA (Q_{HSA}) bound to [PVI^+-silica] as a function of salt concentration

C_{NaCl} (M)[a]	Q_{HSA} (mg/g)	Trp			Ox		
		k_1	k_2	α_{Trp}	k_1	k_2	α_{Ox}
0.2	5	0.2	0.8	4.0	0.75	10	13
0.15	10	0.25	1.7	6.8	1.2	25	21
0.12	20	0.6	7.0	11.5	3.4	–[b]	–[b]

The corresponding retention factors (k) and selectivity values (α) measured for DL-tryptophan (Trp) and *RS*-oxazepam (Ox). Injection volume: 20 μl. Eluent: 0.05 *M* phosphate buffer. Flow-rate: 1 ml/min. Detection at 280 nm.

[a] NaCl concentration during the immobilization of HSA on [PVI^+-silica].

[b] The second enantiomer is not eluted.

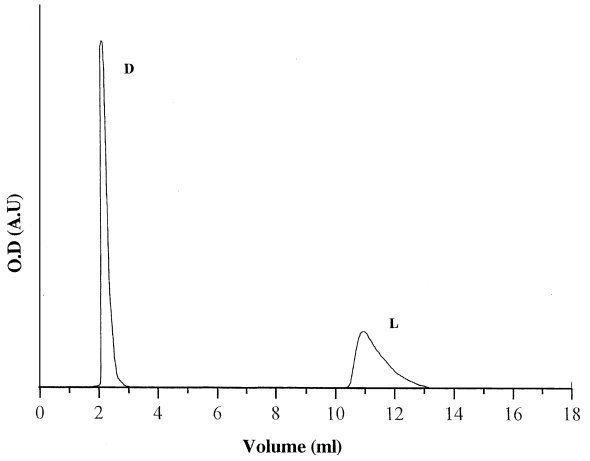


Fig. 1. Elution of DL-tryptophan on [PVI$^+$-HSA]. HSA loading: 20 mg per g of support. Column length: 10 cm. Eluent: 50 m*M* phosphate buffer, pH 7.4. Flow-rate: 1 ml/min.

adsorbed on non-polymeric anion exchangers [18,19]. Thus, it can be assumed firstly that the structure of albumin is preserved by immobilization procedures involving electrostatic interactions. This conclusion was confirmed by an FT-IR study where the structure of HSA bound to PVI$^+$-coated silica was compared to the structure of HSA solutions [20]. It was shown that structural changes for HSA adsorbed on [PVI$^+$-silica] were negligible and concerned less than 3% of the protein backbone.

Moreover, results obtained with [PVI$^+$-HSA] supports demonstrate that the orientation of HSA after immobilization still permits the binding of NBP, tryptophan and oxazepam (warfarin was not eluted under the same conditions). These compounds are known to interact with the indole binding site (site II) on HSA [21–23]. In the case of NBP, it was shown by competitive assays (data not published) that both enantiomers interacted at the same site as L-tryptophan. Earlier studies suggested that Lys-414 in HSA was involved in the binding of L-tryptophan and diazepam (site II) [23]. Since PVI$^+$-coated silicas are anion exchangers, the immobilization of HSA to such supports is achieved via negative residues of the protein. Then, the positively charged Lys-414 is free because it cannot interact with [PVI$^+$-silica] supports. However, Lys-414 could be masked after immobilization of the protein, if it was located in a very negative environment. This hypothesis is in contradiction with the good selectivity observed for the enantiomers of DL-tryptophan, *RS*-oxazepam and DL-NBP. Thus it can be assumed that the indole binding site is still accessible after immobilization of HSA on [PVI$^+$-silica] supports.

The main drawback of stationary phases using proteins adsorbed on ion exchangers is their unstability at high ionic strengths. For example, β-methylaspartase was bound to PVI$^+$-coated silica (154 mg/g). The immobilized enzyme was fully active but was released from the solid support during bioconversions (data not published) which were performed at high salt concentrations [24]. Thus, for applications requiring high salt concentrations, procedures involving covalent grafting of proteins to the matrix must be selected.

3.1.2. Grafting of HSA to silica modified by reactive polymers

Reactive polymers derived from PAC, PMAC and PVOC were evaluated to immobilize HSA onto amino-silica.

After grafting of the polymer to amino groups by means of acyl chloride or chloroformate functions, residual reactive groups were modified by NHS, leading to NHS-ester and NHS-carbonate derivatives, respectively ([PAC-NHS]; [PMAC-NHS]; ([PVOC-NHS]). Then HSA was bound in aqueous media (pH 7.4) to these materials via its amino groups. It appears in Table 2 that maximum amounts of protein immobilized per gram of support were

Table 2
Silica modified by reactive polymers (PAC, PMAC and PVOC) and [PVI$^+$-silica]

	Q_{HSA} (mg/g)	α_{Trp}	α_{NBP}	α_{Warf}	α_{Ox}
[PAC-HSA]	22	2.5	2.4	2.2[a]	5.3[a]
[PMAC-HSA]	18	3.2	2.0	2.4[a]	1.5[a]
[PVOC-HSA]	62	5.2	2.2	1.8[b]	1.5[c]
[PVI$^+$-HSA]	20	11.5	2.0	–	–[d]
	10	7.0	1.9	–	21

Maximum amounts of HSA (Q_{HSA}) and selectivity values (α) measured for DL-tryptophan (Trp) DL-NBP, *RS*-warfarin (Warf), and *RS*-oxazepam (Ox). Injection volume: 20 μl. Eluent: 0.05 *M* phosphate buffer. Flow-rate: 1 ml/min. Detection at 280 nm (except for NBP: 250 nm).

[a] 2% of 1-propanol in the mobile phase.
[b] 6% of 1-propanol in the mobile phase.
[c] 4% of 1-propanol in the mobile phase.
[d] The second enantiomer is not eluted.

lower for [PAC-NHS] and [PMAC-NHS] compared to [PVOC-NHS] phases. NHS-ester and NHS-carbonate functions are known to undergo spontaneous hydrolysis in basic media leading to the formation of carboxylic and neutral OH groups, respectively. Possibly, carboxylate functions formed during the binding of HSA to [PAC-NHS] and [PMAC-NHS] supports may induce repulsive effects towards the negatively charged protein (pI=4.8), decreasing the amounts of HSA in the column.

The resulting [PAC-HSA], [PMAC-HSA] and [PVOC-HSA] stationary phases were evaluated for the separation of racemic mixtures. In the case of *RS*-warfarin and *RS*-oxazepam, 1-propanol (2, 4 or 6%) was added to the mobile phase to elute these compounds from the columns. Unlike [PVI$^+$-HSA] supports, these chiral stationary phases were stable in the presence of organic solvents and at high ionic strengths. As shown in Table 2, they allowed performant chiral separations (Fig. 2A).

Results obtained with [PAC-HSA] and [PMAC-HSA] columns were similar, except for the enantiomers of *RS*-oxazepam which were less resolved on [PMAC-HSA] supports. Since the PMAC backbone is more hydrophobic than PAC polymer chains, PMAC supports probably induce greater changes in the structure of the immobilized protein than PAC-modified silicas. Moreover, the results of competitive binding studies carried out on HSA chiral phases indicated that *R*-oxazepam did not bind at the same site than *S*-oxazepam, D- and L-tryptophan [25,26] and D- and L-NBP (data not published). Thus, it can be assumed that the binding site of *R*-oxazepam is probably more altered on [PMAC-HSA] supports than on [PAC-HSA] phases, resulting in poorer selectivity of [PMAC-HSA] columns for the separation of *R*- and *S*-oxazepam.

It would be hazardous to compare performances of [PVOC-HSA] supports and [PMAC-HSA] or [PAC-HSA] phases because amounts of HSA per gram of support are very different (Table 2). As shown in a previous study [14], selectivity values depended on the protein loading, thus the better selectivity observed for DL-tryptophan with [PVOC-HSA] could be only due to the higher amount of HSA in the column. Moreover, percentage of propanol needed to elute warfarin and oxazepam were higher in the case of [PVOC-HSA] supports. Therefore, α values measured for these compounds cannot be compared on [PVOC-HSA] and [PMAC-HSA] columns, since enantioselectivity of HSA is known to decrease when the amount of organic solvent in the mobile phase increases.

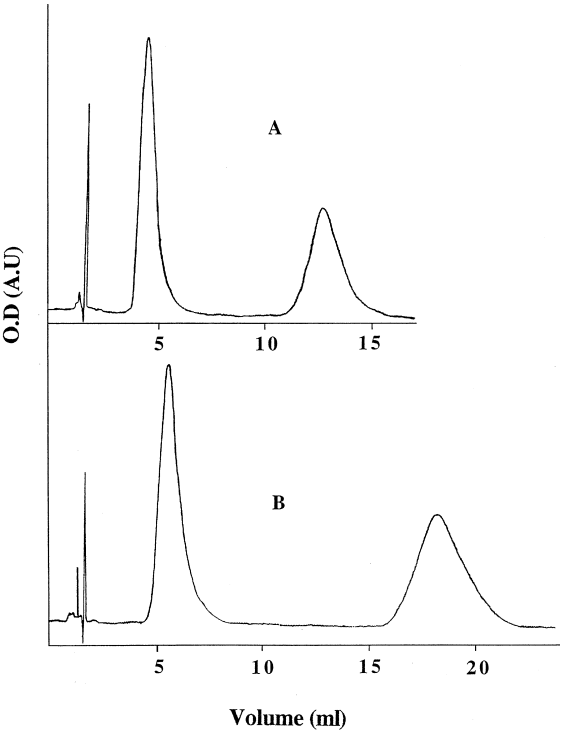


Fig. 2. Elution of *RS*-oxazepam on [PAC-HSA] (A) and [T-PAC-HSA] (B). HSA loadings: 22 mg per g (A) and 24 mg per g (B). Column length: 15 cm. Eluent: 50 m*M* phosphate buffer, pH 7.4 with 5% of 1-propanol. Flow-rate: 1 ml/min.

3.1.3. Retention and enantioselectivity on ionic and covalent chiral stationary phases

[PAC-HSA], [PMAC-HSA] and [PVOC-HSA] supports were compared to [PVI$^+$-HSA] phases. As shown in Table 2, chiral stationary phases using reactive polymers were much less selective for the separation of DL-tryptophan than supports obtained by adsorption of HSA to anion exchangers. Simultaneously, it appeared (Table 3) that L-tryptophan was more retained on [PVI$^+$-HSA] phases (for similar HSA loadings). These results demonstrate that stereoselective interactions were predominant in

Table 3
Retention factors of the enantiomers of DL-tryptophan on [PAC-HSA], [PMAC-HSA], [PVOC-HSA] and [PVI^+-HSA] supports with similar HSA loadings

	[PVI^+-HSA], $Q_{HSA}=20$ mg	[PAC-HSA], $Q_{HSA}=22$ mg	[PMAC-HSA], $Q_{HSA}=18$ mg	[PVI^+-HSA], $Q_{HSA}=60$ mg	[PVOC-HSA], $Q_{HSA}=62$ mg
k_1	0.6	0.5	0.6	0.8	0.8
k_2	7	1.2	2	Not eluted	4.4

Eluent: 0.05 *M* phosphate buffer. Flow-rate: 1 ml/min. Detection at 280 nm.

the retention mechanism of DL-tryptophan on both kinds of chiral supports.

In the case of *RS*-oxazepam, the enantiomers were eluted from [PAC-HSA], [PMAC-HSA] and [PVOC-HSA] columns only after addition of 1-propanol to the mobile phase. For this reason, it was difficult to compare α values obtained with reactive polymers and [PVI^+-HSA] phases, because selectivity strongly decreased in the presence of organic solvents [15]. Nevertheless, from values reported in Table 2, it can be reasonably concluded that [PAC-HSA], [PMAC-HSA] and [PVOC-HSA] supports were less selective for *RS*-oxazepam than [PVI^+-HSA] chiral phases.

For DL-NBP (as for DL-tryptophan), the retention was higher on [PVI^+-HSA] phases than on supports using reactive polymers (data not shown). However, selectivity values for DL-NBP were similar on both kinds of supports (Table 2). This can be correlated to results obtained by competitive binding experiments: it was observed that both enantiomers interacted at the indole binding site and were affected similarly by the addition of L-tryptophan to the eluent (k_1 and k_2 were 25% lower in the presence of 10^{-4} *M* L-tryptophan). Thus it can be assumed that any other modification at the indole binding site (conformational change, modification of the accessibility) will have comparable effects on the retention of D- and L-NBP and result in similar selectivity values.

The lower selectivity observed for DL-tryptophan and *RS*-oxazepam with stationary phases using reactive polymers (Table 2), can be correlated with the presence of an active lysine (Lys-414) at the indole binding site [23]. Proteins are grafted to such reactive supports via their NH_2 groups. Thus, HSA molecules for which Lys-414 is implicated in the bonding become inactive, because their indole binding site is no more accessible to enantiomers. This can explain the lower selectivity of these phases for DL-tryptophan and *RS*-oxazepam compared to [PVI^+-HSA] supports.

Moreover, it was shown by electron paramagnetic resonance (EPR) spectroscopy that reactive polymer grafted to amino silica remained in an entangled state after immobilization, leading to rather compact polymer layers with low mobility [16]. Therefore, the binding of HSA molecules to reactive polymers by several rigid covalent linkages may induce severe protein deformation. On the contrary, electrostatic interactions between the protein and the support are less constraining for the protein structure than rigid covalent linkages. This could be another explanation for the lower enantioselectivity observed in the case of [PAC-HSA], [PMAC-HSA] and [PVOC-HSA] supports compared to [PVI^+-HSA] chiral stationary phases. To circumvent this problem, reactive polymers with flexible arms were evaluated.

3.2. Use of tentacular reactive polymers

Supports with tentacular polymer chains were obtained by in situ polymerization of the monomer on the amino-silica surface. The monomer was first grafted to NH_2 groups of the support via acyl chloride or chloroformate functions. Then the excess of monomer was polymerized in situ, after heating and addition of an initiator to the reaction mixture. It was shown by EPR spectroscopy [16] that polymer chains obtained by this technique undergo faster motion compared to polymer chains grafted by conventional methods. After activation with *N*-hydroxysuccinimide, the maximum amounts of HSA which were bound to [T-PAC-NHS], [T-PMAC-NHS] and [T-PVOC-NHS] phases were slightly higher than for conventional polymer layers (Tables 2 and 4). This probably arises from the mobility of

Table 4
Silica modified by tentacular reactive polymers (T-PAC, T-PMAC and T-PVOC)

	Q_{HSA} (mg/g)	α_{Trp}	α_{NBP}	α_{Warf}	α_{Ox}
[T-PAC-HSA]	24	2.8	1.8	2.1[a]	6.5[a]
[T-PMAC-HSA]	28	5.7	1.9	2.9[a]	2.0[a]
[T-PVOC-HSA]	70	8.4	2.3	1.9[b]	2.0[c]

Maximum amounts of HSA (Q_{HSA}) and selectivity values (α) measured for DL-tryptophan (Trp) DL-NBP, *RS*-warfarin (Warf) and *RS*-oxazepam (Ox). Injection volume: 20 μl. Eluent: 0.05 *M* phosphate buffer. Flow-rate: 1 ml/min. Detection at 280 nm (except for NBP: 250 nm).

[a] 2% of 1-propanol in the mobile phase.
[b] 6% of 1-propanol in the mobile phase.
[c] 4% of 1-propanol in the mobile phase.

polymer chains obtained by this technique which results in a better accessibility to the protein.

The resulting chiral stationary phases allowed performant separation of racemic mixtures (Fig. 2B). As reported in Tables 2 and 4, tentacular supports were slightly more selective than conventional supports. This could be due to the higher HSA loading, except for [PAC-HSA] and [T-PAC-HSA] where protein amounts were nearly the same.

Thus, it can be concluded that tentacular chiral stationary phases are slightly more selective than conventional polymer-coated silicas. They induce less protein deformation because of the mobility of their polymer arms.

However, compared to [PVI^+-HSA] phases, tentacular supports remain less efficient for chiral separations. Thus it can be assumed that the lower enantioselectivity of supports using reactive polymers (conventional or tentacular) mainly results from the reaction of some Lys-414 residues with reactive functions of the supports, which induces a statistical loss of accessibility of the indole binding site. However, conformational effects induced by the immobilization process have to be considered too.

3.3. Influence of the number of linkages between HSA and the support

3.3.1. Binding of HSA in the presence of ethanolamine (EA)

In order to decrease the number of covalent linkages between HSA and the support, as well as the probability of binding via Lys-414, the protein was grafted to [PVOC-NHS] phases in the presence of increasing concentrations of ethanolamine (pH maintained at 7.4). This compound acts as a competitor towards NHS-carbonate functions. As reported in Table 5, selectivity values first increased with the concentration of ethanolamine, then decreased. The phenomenon was very striking for the enantiomers of DL-tryptophan. An analogous effect appeared for *RS*-oxazepam which interacts at the same site than tryptophan (site II) and for *RS*-warfarin which binds to the azapropazone site (site I) [27]. For these compounds, moderate variations were observed because they were analyzed in the presence of 1-propanol.

Since similar results were observed for both kinds of enantiomers, it can be concluded, as expected, that the addition of ethanolamine during the binding of HSA to the reactive polymer probably decreases the number of covalent linkages between the protein and the support. Consequently, the protein is less deformed, leading to more efficient chiral separations. Moreover, in the presence of ethanolamine the number of Lys-414 residues implicated in the binding is probably lower than without competitor. This hypothesis is in good agreement with results observed for tryptophan and at a lower extent for oxazepam (eluted in the presence of 1-propanol).

However, the amounts of HSA bound to the columns decrease at high concentrations of ethanolamine (Table 5), since the number of possible binding centers on the support is lower. Thus the presence of ethanolamine during the binding of HSA has two opposite effects: it induces less structural

Table 5
Binding of HSA to [PVOC-NHS] supports in the presence of ethanolamine

C_{EA} (mol/l)	Q_{HSA} (mg/g)	α_{Trp}	α_{NBP}	α_{Warf}[b]	α_{Ox}[a]
0	62	5.2	2.2	1.8	1.5
$1.2\cdot10^{-3}$	57	8.2	2.4	2.1	1.8
$6\cdot10^{-2}$	46	11.1	2.2	2.0	1.9
$1.2\cdot10^{-1}$	30	9.3	2.1	1.8	1.9

Maximum amounts of HSA (Q_{HSA}) and selectivity values (α) measured for DL-tryptophan (Trp) DL-NBP, *RS*-warfarin (Warf) and *RS*-oxazepam (Ox). Injection volume: 20 μl. Eluent: 0.05 *M* phosphate buffer. Flow-rate: 1 ml/min. Detection at 280 nm (except for NBP: 250 nm).

[a] 4% of 1-propanol in the mobile phase.
[b] 6% of 1-propanol in the mobile phase.

changes of the protein and decreases the number of linkages HSA-support, leading to a better selectivity. Simultaneously, it decreases the amount of protein in the column, resulting in lower α values. These opposite phenomena are responsible for the evolution of selectivity with the ethanolamine concentration (Table 5).

3.3.2. Immobilization of HSA by thiol–disulfide interchange

As shown in the first part of this paper, [PVI^+-HSA] phases were highly selective but could only be used under mild elution conditions. Chiral supports using reactive polymers were stable but less selective than [PVI^+-HSA] phases, as explained before (binding of HSA via its NH_2 groups and to some extent protein deformation resulting from the formation of numerous linkages between the protein and the support). To circumvent these problems, some attempts were made to bind covalently the protein to PVOC-derivatives via its unique thiol group (Cys34) [17]. PVOC-coated silica was modified by a concentrated solution of cysteamine hydrochloride (Reaction 2). IR spectra (spectra not shown) of resulting supports ([PVOC-SH]) showed a band in the carbonyl region assigned to the urethane carbonyl ($\nu_{C=O} = 1700\ cm^{-1}$) while the chloroformate band ($\nu_{C=O} = 1775\ cm^{-1}$) was no longer observable. After activation of [PVOC-SH] by 2,2′-dipyridyl disulfide (2-PDS) (Reaction 3), HSA was bound to the support by thiol–disulfide interchange as described previously in the case of poly(vinylimidazole)-coated silicas [25]. The amount of HSA immobilized by this method was equal to 75 mg per gram of silica. DL-tryptophan and DL-NBP were resolved in phosphate buffer with selectivity values equal to 2.8 and 1.7, respectively. Oxazepam and warfarin were highly retained and were eluted out of the column only after addition of 1-propanol (4%, v/v) to the mobile phase. In spite of the high HSA loading, the enantiomers of *RS*-oxazepam were poorly resolved ($\alpha =$ 1.3). Moreover, *R*- and *S*-warfarin were not separated, suggesting that the accessibility to the azapropazone site [21,22] was hindered by this immobilization procedure. This assumption is in agreement with the illustration proposed by Carter and Ho summarizing the various ligand-binding sites on serum albumin [28].

Compared to [PVOC-HSA] supports (60 mg of HSA per gram), [PVOC-SH-HSA] chiral phases are less selective, although HSA is bound to the polymer via a single covalent linkage involving a thiol group (not a lysine residue). According to Carter and Ho's illustration [28], the indole binding site should be still accessible after binding of HSA via the Cys 34 residue. Therefore, the lower selectivity of [PVOC-SH-HSA] supports is probably due to non-specific interactions between the protein and polymer layer. It can be assumed that residual pyridyl groups are responsible for the observed phenomena since non-modified [PVOC-HSA] phases are more selective than [PVOC-SH-HSA] supports. If numerous contacts indeed, between HSA and the support are established, these interactions can induce conformational changes of the protein. Moreover, interactions can take place at the indole binding site (for instance between pyridyl groups and tyrosine-411 which is implicated in the indole binding site [28]), leading to a loss of accessibility to this site.

Thus, although very promising, [PVOC-SH-HSA] phases were not very performant for chiral separations. They were less selective for the separation of DL-NBP, DL-tryptophan and *RS*-oxazepam than other polymer-coated supports evaluated in this paper. Moreover, although highly retained ($k = 24$), the enantiomers of *RS*-warfarin were not separated. It can be concluded that the azapropazone site is probably masked and that the elution of warfarin is only governed by non-specific interactions with [PVOC-SH-HSA] support

4. Conclusion

Chiral stationary phases obtained by adsorption of HSA on [PVI^+-silica] are much more selective for the separation of DL-tryptophan and *RS*-oxazepam than other materials using reactive polymers. This result is in good agreement with the involvement of an active lysine residue (Lys-414) in the indole binding site. When the linkage of HSA to the reactive polymer occurs at Lys-414, the orientation of HSA no more permits interactions at the indole binding site. Moreover a slight increase in selectivity is observed when the protein is grafted to polymer chains formed by in situ polymerization: due to their

increased mobility compared to conventional polymer layers, there is less protein deformation.

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