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Review

Monoliths with proteins as chiral selectors for enantiomer separation

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ABSTRACT

Recent advances in monolithic systems, especially the development of functionalized monoliths, make it more tentative for separation science, such as enantiomer separation. Proteins, as one category of well-acknowledged chiral selectors, have won a reputation of separating a wide range of enantiomeric mixtures due to varieties of functional groups at their surface. However, the combination of protein and monoliths for chiral analysis is seldom reported, thus the state-of-the-art in monolith with proteins as chiral selectors for enantiomer separations is comprehensively reviewed. This review focuses on organic polymer monoliths, silica monoliths and molecularly imprinted polymer monoliths functionalized by proteins or glycoproteins, introducing the possible mechanisms, the immobilizing ways as well as the associated problems. Related areas such as partial filling technique are also covered.

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Contents

1. Introduction

A lot of chemical compounds used in pharmaceutical formulations feature one or more chiral centers, responsible for optical activity, can strongly impact their pharmacological and pharmacokinetic properties. Therefore, development of new approaches for the separation of chiral compounds is a source of global research efforts and innovative incentives. In addition, the impetus for this

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vibrant activity lies with the increasing pressure and concern from regulatory agencies such as FDA [1].

The broad applicability of proteins as chiral selectors is evident in the large number of racemates separated so far, and is further expressed by simple adsorption or immobilization to the stationary phase for inducing a reversible change of the selector conformation, thus obtaining different enantioselective properties of the same protein. Because of their chiral nature and the variety of functional groups present at their surface, proteins can interact with chiral entities by forming not only relatively weak and non-specific bonds, but also stronger and more specific interactions [2,3], which will be discussed specifically.

In the past decade, monolithic separation media [4] have become a preferred column packing material for microscale separation techniques in capillaries and columns [5–8]. Advantages such as ease of in situ preparation or narrow channels of micro fluidic devices [9] from reaction solutions of monomers or reactive precursors make them tentative to ideal stationary phases for various separation formats. Different types of monolithic materials have been developed, which partly evolved from less successful pioneering studies of others sometime earlier [4,10]. Among them, organic polymer monoliths emerged in the late 1980s in the form of highly swollen cross-linked polyacrylamide gels, first suggested by Hjertén [11], and rigid cross-linked polymethacrylate copolymers proposed by Tennikova et al. [12]. Other types of organic polymer monoliths appeared later such as polystyrene monoliths, monoliths prepared by ring-opening metathesis polymerization (ROMP), and monoliths from poly-condensation, which have been described in detail by a recent review of Svec [10]. Apart from organic polymer monoliths, silica monoliths, although not so widely applied at the beginning, have been developed based on sol–gel technology by Nakanishi and Tanaka in the mid 1990s [13], accelerating the development of silica monoliths. Both types of preparation will be elaborated later in the field of enantio-separation using proteins as chiral selectors.

In this review, proteins as chiral selectors for the separation of enantiomers include: albumins such as bovine serum albumin (BSA) [14] and human serum albumin (HSA) [15]; glycoproteins such as α-acid glycoprotein (AGP) [16], ovomucoid from chicken egg whites (OMCHI) [17], ovoglycoprotein from chicken egg whites (OGCHI) [18], avidin (AVI) [19], and riboflavin binding protein (RfBP) (or flavoprotein) [20]; enzymes such as trypsin [21], achymotrypsin [22], cellobiohydrolase I (CBH I) [23], pepsin [24], and lysozyme [25]; other proteins such as ovotransferrin (or conalbumin) [26] and β-lactoglobulin [27]. However, for the use of chiral selectors in monoliths, not all of proteins described above have been applied as immobilized ligands or encapsulating compounds.

2. Protein selectors

2.1. General view

A protein and glycoprotein, both of which are chiral, have the possibility to discriminate a chiral molecule. However, only a limited number of proteins have been investigated as chiral selectors, not to mention binding them on monoliths. The physical properties of proteins used as chiral selectors are shown in Table 1. The most extensively investigated protein ligands in chiral monoliths are human serum albumin (HSA), bovine serum albumin (BSA), and α-acid glycoprotein (AGP) [28], which can be concluded through Table 2 from summarization at the end of this review.

Due to the structural complexity of these macromolecular protein selectors, their chiral recognition mechanisms at the molecular level remained unknown for a long time. However, with the advent of modern technology, like protein NMR [29], X-ray crystallography [30] and docking studies [31], the mysterious mechanisms for chiral recognition are being unveiled. The binding modes have become known for a number of protein–guest complexes [32].

2.2. α-Acid glycoprotein

α-Acid glycoprotein is one of glycoproteins used in chiral monoliths. AGP is the major plasma protein responsible for the protein binding of cationic drugs because AGP has a lower isoelectric point (p*I*) value than BSA and HSA (Table 1).

Hage and co-workers [33,34] bound AGP via its carbohydrate chains after periodate oxidation to hydrazide-activated supports. Silica particles, silica monoliths and polymer monoliths based on glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) were used as the support. The surface coverage of AGP in the silica monolith is 18% higher than that obtained with silica particles and 61% higher than that with a GMA/EDMA monolith. The higher surface area of the silica monolith gives materials that contain 1.5–3.6 times more immobilized protein per unit volume when compared [34].

Besides the high heterogeneity of its glycans, the protein part of AGP has also shown polymorphism. The variants are encoded by two different genes: the F1–S variants are encoded by the alleles of the same gene, while the A variant is encoded by a different gene [35]. There is a difference of at least 22 amino acid residues between the F1–S (ORM 1) and A (ORM 2) variants, while F1 and S forms differ

Table 1
Physical properties of proteins as chiral selectors.

Protein[a]	Molecular mass (kDa)	Isoelectric point	Carbohydrate proportion (%)	Origin
Glycoproteins				
AGP	44	2.7	45	Human or bovine serum
OMCHI	28	4.1	30	Egg white
OGCHI	30	4.1	25	Egg white
AVI	77	10.0	7	Egg white
Streptavidin	66	7.0	–	Bacterium
RfBP	32–36	4.0	14	Egg white
Albumins				
BSA	66	4.7	–	Bovine serum
HSA	65	4.7	–	Human serum
Enzymes				
Fungal cellulase	60–70	3.6	6	Fungus
CBH I	64	3.9	6	Fungus
Lysozyme	14.3	10.5	–	Egg white
Pepsin	34.6	<1	–	Porcine stomach

[a] AGP: α-acid glycoprotein; OMCHI: ovomucoid from chicken egg whites; OGCHI: ovoglycoprotein from chicken egg whites; AVI: avidin; RfBP: riboflavin binding protein; BSA: bovine serum albumin; HSA: human serum albumin; CBH I: cellobiohydrolase I.

Table 2
Summarization of monoliths with proteins as chiral selectors.

Monoliths backbone[a]	Types of protein[b]	Analytes	Ways of binding	Reference
Commercial silica monolith	AGP	Warfarin, propranolol	hydrazide immobilization method	[33,34]
GMA/EDMA	HSA	D,L-Amino acids, tryptophan	Epoxy means, Ethylene Diamine means	[86]
TMOS/MTMS	OVM, BSA	Tryptophan, benzoin, eperisone, chlorpheniramine	Encapsulation through sol–gel process	[89–92]
Commercial silica monolith	trypsin	Protein digestion and peptide analysis	Epoxy means	[93]
GMA/EDMA	HSA	Warfarin, tryptophan	Epoxy means	[94]
Commercial silica monolith, GMA/EDMA, silica particles	HSA	Warfarin, tryptophan	Epoxy, mild reduction	[95]
Commercial silica monolith	HSA	Warfarin	sulfhydryl immobilization method	[97]
Commercial silica monolith	trypsin	Protein digestion and peptide analysis	Epoxy means	[99]
Dimethylaminopropylmethacrylate, tetraethylene glycol dimethacrylate	Cytochrome c, lysozyme, myoglobin	–	Imprinting method	[115]
Imprinted polymers by the combined use of chitosan	bovine hemoglobin	–	Imprinting method	[120–122]
Metal surface and polymer	urease and BSA	–	Imprinting and coating	[124–126]
Microbeads	BSA	–	Coating imprinted thin film	[127]
Hybrid silica-based MIP monolith	BSA and lysozyme	–	Imprinting method	[134]
–	Canine serum albumin, BSA	Tryptophan derivatives	Partial filling	[138]
–	Penicillin G acylase	Ketoprofen	Partial filling	[139]
–	HSA	Bupivacaine	Partial filling	[140]
–	HSA	Promethazine, trimeprazine	Partial filling	[141]
–	HSA	Five antihistamines	Partial filling	[146]

[a] GMA: glycidyl methacrylate; EDMA: ethylene glycol dimethacrylate; TMOS: tetramethoxysilane; MTMS: methyltrimethoxysilane; MIP: molecularly imprinted polymer.
[b] AGP: α-acid glycoprotein; HSA: human serum albumin; OVM: ovomucoid; BSA: bovine serum albumin.

only in a few residues. Selective binding of coumarin enantiomers to human AGP genetic variants was investigated. All investigated compounds bound stronger to ORM 1 than to ORM 2 [36]. ORM 1 and human native AGP prefer the binding of (S)-enantiomers of warfarin and acenocoumarol, while no enantioselectivity is observed in phenprocoumon binding. Ligand-binding properties of AGP are also investigated by circular dichroism (CD) methods, from which drug–AGP complexes were observed with several class of drugs [37].

2.3. Human serum albumin and bovine serum albumin

Human serum albumin (HSA) has been thoroughly investigated owing to its important role as drug transporting plasma protein, which is also proved to be effective for the separation of enantiomers [38]. Enantioselective determination of bupivacaine, oxprenolol, and propranolol in pharmaceuticals through HSA has been reported [39]. One of the main advantages of using HSA is the low cost per analysis (0.006 D/run), since HSA solution is not electrolyzed and can be reused for several runs [40].

Of several complexes of HSA with drugs or toxins, X-ray crystal structures have been discovered and are available via the Brookhaven protein data bank [41], which can be seen of existing two primary binding sites for drugs and a number of secondary ones where drugs can bind with varying specificity. Of particular interest from a viewpoint of chiral recognition are X-ray crystal structures reported for warfarin because both of the diastereomeric complexes are available (Fig. 1) [42]. Warfarin binds to the subdomain IIA and as can be seen, both R- and S-enantiomer bind in the pocket in almost identical conformations and geometric arrangement. Coumarin and benzyl-moieties of the R- and S-form are nearly perfectly superimposable in overlaid complexes. The main difference in the drug is conformations in the acetonyl group and to H-bond interactions that are formed between Arg222 residue and the carbonyl of the coumarin ring (in R-complex) and of the acetonide (in S-complex) [43]. The enantiomers bind in essentially the same way to HSA is consistent with the observation that they have similar binding affinities for the protein which is thus characterized for a low degree of enantioselectivity for warfarin enantiomers.

Affinity capillary electrochromatography (CEC) with zonal elution method is used to probe the competitive interactions of enantiomers with bovine serum albumin (BSA) [44]. The binding sites of solutes on the BSA molecule are determined by the changes in the retention factors of the solutes resulted from the addition of competitive agent. By using D- or L-tryptophan as competitive agents and D-, L-tryptophan and benzoin enantiomers as injected analytes show that BSA molecule has a primary site to strongly bind L-tryptophan, but D-tryptophan dose not bind at this site; D-and L-tryptophan share a weak binding site on the BSA molecule. Benzoin enantiomers do not share any binding sites with either D- or L-tryptophan. Non-chiral compounds of trichloroacetic acid and n-hexanoic acid are applied as the competitive agents to study the binding of warfarin enantiomers to BSA, and it is observed that trichloroacetic acid and n-hexanoic acid have a same binding site for warfarin enantiomers binding to BSA molecule [44].

2.4. Ovoglycoprotein from chicken egg whites

OGCHI consists of 203 amino acids including a predictable signal peptide of 20 amino acids. The mature OGCHI shows 31–32% identities to rabbit and human AGPs. Thus, OGCHI should be the chicken AGP (cAGP), a member of the lipocalin families [45]. Tanaka [46] found that the OMCHI used in previous studies was crude and they isolated a glycoprotein from chicken egg whites and termed it OGCHI. It is found that about 10% OGCHI is included in crude OMCHI preparations, and that chiral recognition ability of OMCHI reported previously [47] comes from OGCHI and pure OMCHI has no chiral recognition ability.

Chicken 1-acid glycoprotein (1-AGP) consists of 183 amino acid residues and has only one Trp residue at the 26 position. Matsunaga [48] modified the Trp26 residue with 2-nitrophenylsulfenyl chloride and examined chicken 1-AGP and Trp-modified chicken 1-AGP columns for the chiral separation of neutral, acidic and basic compounds. Chiral separation of propranolol, alprenolol and oxprenolol was lost on the Trp-modified chicken 1-AGP column, while chlorpheniramine, ketoprofen and benzoin were still enantioseparated on the Trp-modified chicken 1-AGP column despite of lower

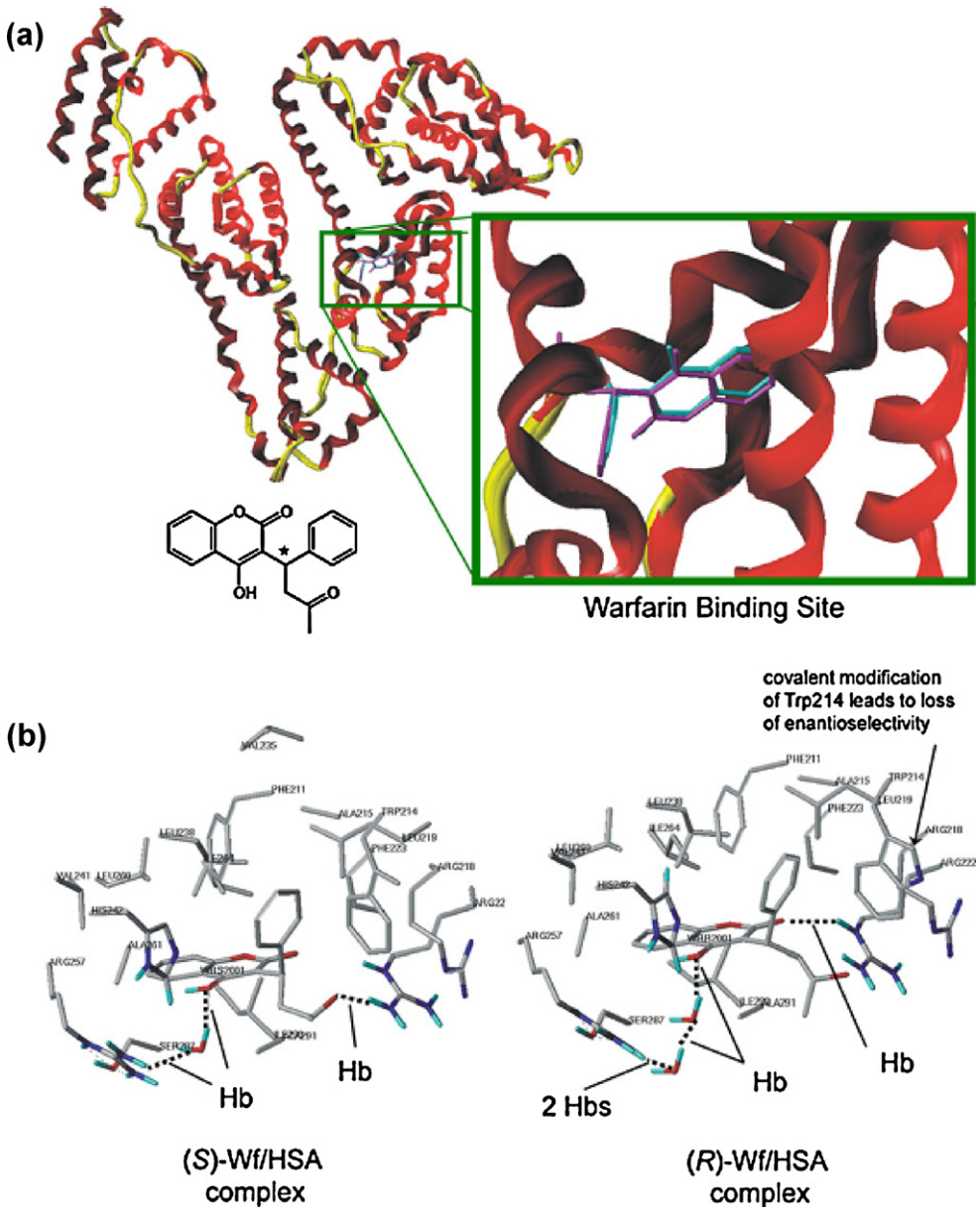

Fig. 1. X-ray crystal structures of HSA–warfarin (Wf) complexes [42]. (a) Superimposed complexes, (R)-Wf (magenta), (S)-Wf (cyan) and (b) active sites with binding modes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

enantioselectivity than that on the chicken 1-AGP column. These results suggest that the Trp26 residue could be responsible for chiral recognition of these compounds [49].

2.5. Riboflavin binding protein and avidin

Riboflavin binding protein (RfBP), through which some acidic and basic racemates were resolved [50], is not very abundant in either the egg yolk or in the egg white of avian species [51]. As the amount of protein required to produce a column is relatively large, an optimization of the purification process becomes necessary. In order to obtain approximately 300–400 mg per batch, four types of RfBP have been described as chiral selectors so far, namely chicken egg yolk (cYRfBP) [52], quail egg yolk (qYRfBP) [53], quail egg white (qWRfBP) [54] and chicken egg white (cWRfBP) [55].

FABPs belong to a family of low molecular mass proteins, exhibiting high affinity binding constants for small endogenous and exogenous lipophilic ligands. Chiral stationary phases based on FABP from chicken liver were successfully applied to enantioseparations of some aryl- and aryloxypropionic acids (suprofen, flurbiprofen, 2-(3-ethylphenoxy)propionic acid, 2-(3-phenyl-phenoxy)propionic acid, 2-(3-phenoxyphenoxy)propionic acid and 2-(2,6-dimethylphenoxy)propionic acid [56]).

Avidin and streptavidin, extracted likewise from egg yolk, have very similar primary structure. They both are tetrameric proteins, isolated from hen egg white and from the bacterium *Streptomycin avidinii*, with isoelectric point 10 and 5, respectively. The streptavidin-based stationary phase has been used for the separation of adenosine enantiomers [57].

Liu et al. [58] studied with avidin as a chiral stationary phase prepared by the physical adsorption on a silica based monolith. Enantiomeric separations of amino acid derivatives, several organic acids, menadione sodium bisulfite, warfarin and N-methylpseudoephedrine were achieved in both nano-HPLC and CEC mode. Theoretical plate numbers of 122,000 per meter for nano-HPLC and 242,000 per meter for CEC were observed.

2.6. Penicillin G-acylase

PGA of *Escherichia coli* catalyzes the hydrolysis of penicillin G to phenylacetic acid and 6-aminopenicillanic acid [59]. PGA is immobilized via its amino and carboxyl groups [60], which the former is attained using amino-silica gels activated by N,N′-disuccinimidylcarbonate (DSC) and epoxy-silica gels, and the latter is using amino-silica gels with 1-ethyl-3-(3′-dimethyl-aminopropyl)carbodimide and N-hydroxysulfosuccinimide. The best results in terms of bound amount of PGA, enzymatic activity and enantioselectivity are obtained with PGA-immobilized on epoxy-silica particles. Enantiomers of 2-arylpropionic acid derivatives such as ketoprofen, suprofen and fenoprofen, and phenoxypropionic acids such as 2-(4-phenoxyphenoxy)propionic acid and 2-(4-benzylphenoxy)propionic acid are separated on PGA-based columns [60]. Furthermore, PGA is immobilized on epoxy-silica monoliths [61]. Silica particle- and monolith-based CSPs are compared with their enantioselectivity. The former gives the higher resolution than the latter, if considering that the amount of bound PGA is lower in the former stationary phase. The worse resolution values on the latter column are the consequence of the lower selectivity unlike the efficiency. A possible explanation could be that the higher protein coverage on the PGA monoliths determines an increase of the non-specific interaction whereas, on the other hand, hindering the access to the specific catalytic site. Furthermore, chiral recognition mechanism on PGA is investigated using molecular modeling and docking studies [62–66], similar to the ways of AGP and HSA.

2.7. Cellobiohydrolase and glucoamylase

Some enzymes used in chiral separation include fungal cellulase from fungus *Aspergillus niger*, CBH I from fungus *Trichoderma reesei* [67], pepsin from porcine stomach [68] and lysozyme from egg whites [61]. The fungal cellulase from different organisms is used in chiral separation. Most of applications are performed using CBH I, which is widely used in HPLC stationary phases. CBH I has a structural organization with a terminal, 36 residue-long binding domain connected to the rest of the enzyme (i.e., the core) through a flexible arm. The core is enzymatically active. The dominating enantioselective site for solutes is located on the core, the main part of the enzyme. The three-dimensional structure of the active site of CBH I has been elucidated by X-ray crystallography, and it has been shown that the binding site is a 40 Å long tunnel [69]. Some basic compounds, especially β-blockers, could be resolved in chiral capillary electrophoresis based on fungal cellulase and CBH I. Pepsin is useful for the enantioseparation of basic compounds, while lysozyme is used for tryptophan and phenylthiohydantoin (PTH)- or dansyl-amino acid. Detailed knowledge from X-ray crystal structure analysis about the molecular interaction mechanism is also available for cellobiohydrolase (CBH) in complex with (S)-propranolol [70]. Chiral stationary phases based on glucoamylases G1 and G2 from *A. niger* have been introduced by Karlsson and his co-workers [71,72]. More information about mechanisms of proteins that have been used as chiral stationary phases can be found in the review published by Haginaka in 2008 [73].

2.8. Lipophilicity of chiral selectors

Barbato [74] investigates ten quinolone antibacterial agents on HSA or AGP column. Among ofloxacine and flumequine, the two chiral compounds in the selected set, only the latter show a split chromatographic peak and only on HSA but not on AGP, indicating that enantioselective specific sites play only a minor role in the retention. With various lipophilicity scales, the interaction of the more lipophilic quinolones, mainly the zwitterions, with serum proteins is not governed uniquely by lipophilicity but also by other mechanisms, probably of electrostatic nature [74].

Later, the same group published another investigation on thirteen blockers, who possess at least one chiral center, using the same method with the same lipophilicity scales [75]. It is found that eight blockers are enantiomerically resolved on the AGP column whereas only four blockers are resolved on the HSA column. Moreover, interactions between blockers and AGP are much stronger than those with HSA. Retention values on both HSA and AGP for less retained enantiomers relate well with various lipophilicity parameters. Different from noctanol lipophilicity values, these values encode both lipophilic. Electrostatic intermolecular recognition forces which may be involved in the interaction between ionized analytes, such as blockers and proteins. However, their effectiveness to describe non-specific interactions with serum proteins for other classes of drugs needs further investigations. Analyses performed on AGP with eluent containing dimethyloctylamine (DMOA) as the displacer demonstrated that enantioselective sites bind to both (−)-forms and (+)-forms, but the binding to (−)-forms is stronger. The enantiomer competition to bind to a same site may be relevant from a pharmacokinetic point of view when racemic mixtures are administered. Finally, in contrast to previously reported data in the literature [76], they found that AGP can bind enantioselectively not only the more lipophilic congeners but also the less lipophilic ones. However, it should be underlined that blockers are structurally related compound with a similar ionization degree; therefore, this conclusion cannot be extrapolated to other classes of compounds.

3. Monoliths

3.1. General remarks

Traditionally, the preparation of column with protein as chiral selector is either through direct coating or through particles carrying chiral selector. Direct covalent binding of BSA to the internal surface of a capillary for enantiomer separation belongs to the former category [77]. These capillaries are operable up to one year when stored properly at 48 °C [77], but this form of easy desorption property is well known. Thus, using silica particles carrying a chiral selector becomes another option. The packing bed is retained by frits at both ends of the capillary, which is complicated and shows limited reproducibility. The problem here is that frits are a source of air bubbles and they break easily.

To circumvent these problems, columns consisting of a block of a porous solid, called monolith or rod, prepared on a silica base either by a sol–gel process using polycondensation of alkoxysilanes or by polymerization of organic monomers, are developed. The essential advantages of monolithic columns come from the possibility to optimize proportions of monomers and cross-linkers so as to control the average size of the throughput channels and the porons. More and more analysts have sensed these advantages, and have achieved a lot of groundbreaking findings in this area, especially the monolithic applications in separation science. Therefore, there is no wonder that reviews on the preparation of monoliths with chiral surface flourish in recent years [78–82]. However, as far as we know, no review is exclusively focused on the monoliths with protein as chiral selectors. In this section, the preparation of monoliths with protein as chiral selectors would be introduced in detail, and we hope this article can illuminate those who are interested in preparing “protein” monoliths.

3.2. Polymer-based monoliths

Early developments of stereoselective capillary electrophoresis with gel-filled columns or completely homogeneous separation media with BSA physically or chemically incorporated in slightly cross-linked neutral gel matrixes may be regarded as pioneer works [83]. The challenge in the course of development of enantioselective organic polymer-type monolithic capillary columns is to get a grasp on the complex interrelations between chiral recognition, EOF control, non-specific adsorption, retention balance, rigidity and permeability without negative mutual effects [84]. This may be the reason why so few recent published articles about fabricating polymer-based monolith with protein as chiral selector could be found, even though more preparation studies of polymer-based monoliths are discussed and developed, including different monomers, porogens, and ways of polymerization [85].

A new kind of immobilized HSA column based on poly(GMA-EDMA) as the support of high-performance affinity chromatography [86] is one of them. Using the epoxide functional groups presented in GMA, the HSA immobilization procedure was performed by two different means: epoxy means and EDA (ethylene diamine) means. The monoliths are successfully adopted for the chiral separation of D,L-amino acids (AAs) and are shown to be applicable to the quantitative analysis of D-tryptophan and are used for the analysis of urine samples [86]. Despite no significant difference between two immobilization means has been observed either in the separation results or stability of the prepared protein columns, the process of EDA means was quite complicated and time-consuming. Moreover, another disadvantage of this immobilization means is a potential for production of undesirable by-products, like homoconjugates and various polymers [87]. Therefore, the simpler epoxy means is more frequently applied.

3.3. Silica-based monoliths

Macroporous polymers based on GMA and EDMA have been employed in many studies to create affinity monoliths. However, the relative large number of publications in which affinity ligands have been used with silica monoliths is surprising since these supports offer several potential advantages. One of these possible advantages is the high surface area of these materials, which would be expected to allow for a high level of immobilized protein attachment. Another expected advantage of silica monoliths is their ability to use the same immobilization methods with these supports that are employed when attaching different affinity ligands to silica particles [88].

Enantioselective silica monoliths are commonly obtained by post-functionalization with chiral selector sites. Therefore, not many attempts have been made to prepare chiral silica-based monoliths in situ by single-step concepts. Protein-encapsulation into a sol–gel matrix during its formation constitutes this in situ preparation approach [89–91]. BSA or ovomucoid (OVM) was add-mixed to a fully or partially hydrolyzed silica precursor, injected into the capillary and the protein finally trapped in the silica network. In the initial study, successful separations of Trp could only be obtained with a gel which was made from TMOS and MTMS, but not with gels made from TMOS alone. Later, Kato et al. [91] developed a novel sol–gel method, encapsulating BSA or OVM into TMOS-based silica matrix in a single step within a capillary. Because no further thermal treatment was performed, a monolith composed of a TMOS-based hydrogel was formed without shrinking. Column fabrication also involved an aging step of the gel for about three days after which the proteins were completely encapsulated in the gel network. Enantiomeric separation of tryptophan and benzoin was achieved on a BSA-encapsulated monolith and the enantiomers of eperisone, chlorpheniramine and benzoin were resolved on an OVM-encapsulated monolith. Under optimized conditions, theoretical plate number for the first eluted enantiomer of benzoin reached 72,000 plates per meter.

While the run-to-run repeatability was quite satisfactory, the lifetime of the monolithic capillary was a problem due to the loss or denaturing of the proteins. In a further paper of the same group [90], BSA-encapsulated monoliths were characterized by their attenuated total reflectance-FT-IR (ATR-FT-IR) [32] and a similar methodology was employed by using the natural polymers gelatine or chitosan as copolymers during the sol–gel process, a more stable BSA-encapsulated silica monolith with somewhat higher enantioselectivity generated [92].

The preparation of a silica monolith for the immobilization of HSA was also examined, using the epoxy immobilization method for attaching proteins to silica monoliths [93]. However, it is known that the epoxy method tends to give lower activities and lower protein coverage for HSA than other amine-based coupling methods [94]. Work in Rangan's study [95] used the Schiff base, an immobilization method which gives better results for HSA when used with other support materials [96]. $NaBH_4$ phosphate solution (pH 8.2) is used to reduce double bond C=N to single bond C—N, which may pose great danger to researchers' life. A mild reducing agent (sodium cyanoborohydride) was also present during this reaction to reduce the Schiff base to a more stable secondary amine linkage [95], which is illustrated in Fig. 2.

Through another way, HSA is immobilized via its sulfhydryl groups [97]. Amino-silica is activated by succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) followed by reaction with a sulfhydryl group of the protein. Similarly, amino-silica is activated by succinimidyl iodoacetate (SIC) for reaction with a sulfhydryl group. Maleimide-activated silica (the SMCC method) or iodoacetyl-activated silica (the SIA method) is used for these methods. It is found that the SMCC and SIA methods

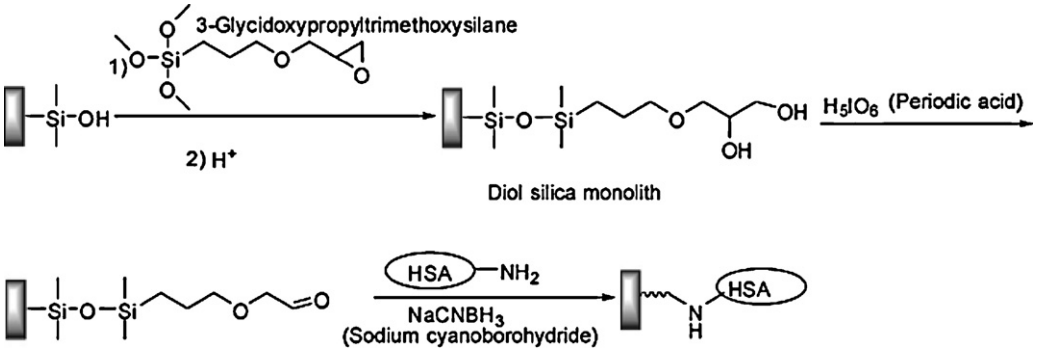

Fig. 2. Reactions for the preparation of an HSA silica monolith [34]. Abbreviation: HSA, human serum albumin.

gave HSA-based monolith with comparable or improved activity and stability, compared to those made by the Schiff base method.

Often, the preparation of a silica monolith for the AGP selector has been immobilized onto epoxy-activated supports [98,99] or through the hydrazide immobilization method, a technique that has been shown to give site-selective coupling for glycoproteins like AGP, as demonstrated in previous work with silica particles [33,100]. Prior to immobilization, the carbohydrate residues on AGP were oxidized under mild conditions to generate aldehyde groups, approximately, five reactive aldehyde groups per AGP molecule [101].

There have been several attempts to prepare particle-based monolith, using silica particles bearing a chiral selector without the need for end frit preparation. In all cases, the immobilization of the packing material inside the capillary requires one additional preparation step. Such capillaries have been obtained, for example, by packing silica material into a tube followed by subsequent sintering of the whole packing material [102], by passing a sol solution of silicate [103] or alkoxysilanes through a pre-packed column [104], or by pumping a methacrylate solution through the column prior to polymerization [105]. Another attempt to prepare particle-based capillaries without end frits was published by Kato et al. [106]. They prepared particle-loaded phases using a sol–gel technique by suspending silica particles containing a chemically bonded chiral selector in a solution of tetraethyl orthosilicate, which was forced into a piece of tubing. Prepared particle-loaded monoliths were characterized by means of electron microscopy. An almost homogeneous distribution of silica particles could be achieved by various optimizations of the polymerization mixture used. Interestingly, silica particles were only slightly cross-linked by the polymeric backbone, leaving the majority of the silica particle surface unaffected. As chiral separation takes place only at the surface of silica particles bearing the chiral selector, this should lead to good separation performance.

3.4. Molecularly imprinted polymer monoliths

Molecularly imprinted polymers (MIPs), another popular technique for enantioselective binding-sites creation, have a potential in the separation of chiral compounds, predicting not only the recognition ability but also the elution order [107–109]. Applications of MIP as separation media in LC, CE and CEC for chiral separation have been extensively investigated [110,111]. The MIP monolith of CEC has the advantage of minimal chemical consumptions, especially the imprinted molecule.

To date, the imprinting of small molecules has been well-established and considered almost routine. However, the imprinting of biomacromolecules, such as proteins and peptides, continues to be a significant challenge due to difficulties with large molecular sizes, structural complexity, environmental sensitivity of the templates, and significantly reduced non-covalent template monomer interactions in aqueous media [112–114]. Despite these difficulties, there is still a strong incentive to synthesis MIPs of biomacromolecules and quite a few attempts have been made with more or less success.

For example, an approach using systematic optimization for the formation of an albumin MIP has been prepared by imprinting albumin using a copolymer comprising 3-dimethylaminopropylmethacrylate and tetraethylene glycol dimethacrylate in a mole ratio of 1–8. Cytochrome c, lysozyme and myoglobin were used in competitive rebinding experiments to compete with the polymer's native template with all protein species present at 0.0004 g/mL. It has been investigated that 0.125 mole ratio of monomer to crosslinker, 6.04 wt.% water content with respect to the mass of the monomer complex, 60 h polymerization time at 38 °C, and with 0.47% albumin in the prepolymerization monomer complex can obtain the optimum condition [115].

Three major challenges can be encountered for MIP taking proteins as template. First challenge is diffusional limitations in large molecular weight for molecularly imprinted polymers, which is attributed to the high crosslinking required to achieve recognition. With increased crosslinking, large templates such as proteins can become entrapped in the network after polymerization. If the template molecule cannot be extracted, the network is rendered useless for bulk recognition applications [116]. In addition, high crosslinking can decrease diffusion by these molecules into the network and lead to longer times needed for recognition.

Another problem is the difficulty of protein dissolution in the monomer solution and the low solubility of these structures in non-aqueous solvents. As proteins increase in complexity, the solubility of these molecules in non-aqueous solvents decreases [117]. BSA is an intermediate-sized (60 kDa), biologically relevant protein that is extremely soluble in water (4100 mg/mL). However, in methanol, the solubility drops to less than 0.05 mg/mL, and the addition of other molecules such as monomers or salts will continue to decrease BSA's solvation by non-aqueous solvents [118,119]. In addition, proteins dissolved in organic solvents exhibit distinctly different biological activity when separated and redissolved in aqueous media [117]. This change in activity is attributed to a conformational change in the protein due to unfolding and refolding in different media. This conformational change can have profound effects on the biological activity of protein. Therefore, it seems that aqueous solvents are the most relevant reaction solvents for MIP networks in order to imprint the correct conformation for recognition in aqueous media. However, as noted previously, polar solvents such as water can interfere with monomer–template interactions, so this challenge is daunting and can help explain the limited research in using proteins as a template in MIP.

Most of the protein-imprinted polymers demonstrated to date show very low template rebinding, which forms the third challenge. Few exhibit rebinding capacity high enough for some applications such as bioseparation. Among these, Guo's group [120–122] fabricated bovine hemoglobin (Hb)-imprinted polymers by the combined use of chitosan, presenting a high Hb rebinding capacity of above 20 mg/g wet gels, while the non-imprinted polymers (NIPs) bound very little Hb. However, Guo found that these high binding results are difficult to explain reasonably. Through X-ray diffraction and scanning electronic microscope investigations, a remarkable property changes of the NIPs after the washing process is confirmed. These findings indicate that the non-specific adsorption resulting from the template removing process rather than the imprinted sites generated on the MIPs themselves may account for the high template rebinding capacity of the reported protein-imprinted polymers [123].

In order to conquer such difficulties, a series of techniques are proposed to be more easily removed and recognized. Surface imprinting techniques is one of them. Ratner and colleagues [124] have developed a way of depositing proteins on metal surfaces and then coating the material with a simple carbohydrate that non-covalently complexes with amino acid residues of the protein. The carbohydrate is then polymerized in the presence of a crosslinker to create a polymer with specific surface recognition sites for the protein. However, few other groups have attempted bulk imprinting within gels; although one group has made a success in imprinting albumin in microgram quantities for sensor applications [125]. Another group successfully imprinted both urease and BSA with polysiloxane but could not universally apply it to myoglobin or hemoglobin [126].

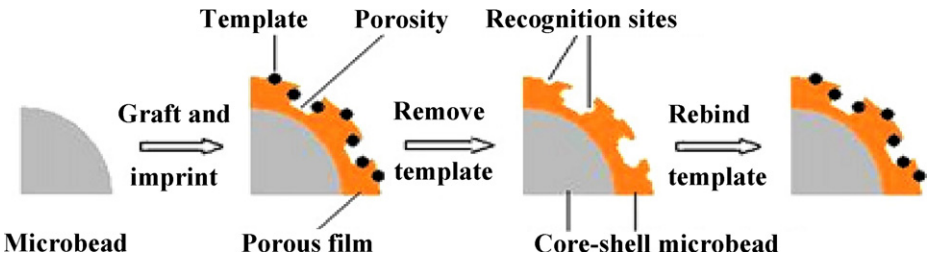

Fig. 3. Schematic illustration of the surface imprinting technique [127].

To make biological macromolecular MIPs possess enough effective area and good property of mass-transport, the technique of coating imprinted thin film on the surface of microbeads has been successfully utilized [127]. The molecule imprinting technique can create a functional film on the surface of organic microbeads and form so-called core–shell material (Fig. 3). First, microbeads as the cores were prepared by styrene polymerizing, then imprinted thin films of porous pAPBA as the shells were grafted onto the surface of microbeads in aqueous solution. After removing template, recognition sites situated at the surface have specific rebinding ability for template proteins.

The development of MIP monolithic column is another alternative approach that may overcome these disadvantages to a great extent. MIP monoliths have rapidly evolved in recent years [128–130], since Matsui et al. [131] employed an in situ polymerization technique to prepare MIP monolithic rods in 1993. Depending on the monolithic material, the MIP monolithic matrix can be divided into organic polymer-based and silica-based monolith. In comparison of a host of polymer-based MIP monoliths used for enantioseparation, only few publications [132] reported on the preparation of a silica-based MIP capillary monolithic column for enantioseparation of small molecules since the sol–gel process is difficult to control and often associated with the cracking and shrinking of silica skeleton during drying. To solve these difficulties, Wang et al. [133] have developed an attractive method for fabrication of a hybrid silica-based MIP capillary monolith based on room temperature ionic liquid-mediated, non-hydrolytic sol–gel process, with which chiral separation of naproxen has been achieved by capillary electrochromatography. Lina [134] further focused on the development of a novel hybrid silica-based MIP monolith technology and its first application in protein recognition. The macroporous silica-based monolithic skeleton was synthesized in a 4.6 mm i.d. stainless steel column by a mild sol–gel process with methyltrimethoxysilane (MTMS) as a sole precursor, and BSA and lysozyme (Lyz) were representatively selected for imprinted templates. Under the optimum conditions, the obtained hybrid silica-based MIP monolith showed higher binding affinity for template than its corresponding non-imprinted (NIP) monolith. The imprinted factor (IF) for BSA and Lyz reached 9.07 and 6.52, respectively. Moreover, the hybrid silica-based MIP monolith displayed favorable binding characteristics for template over competitive protein. Compared with the imprinted silica beads and in situ organic polymer-based hydrogel MIP monolith, the hybrid silica MIP monolith exhibit better chromatographic performances [134].

The combination of MIP with chiral separation through macromolecules, at early times, only came from papers comparing the prepared MIP with commercially available chiral stationary phases. For example, the baseline separation of nilvadipine enantiomers through MIP was compared with columns based on the protein (ovomucoid or α-acid glycoprotein) [135].

However, apart from applications of bioseparation and biosensors, no imprinting of protein has been applied for chiral separation. This may due to that macromolecular MIP is so difficult to fabricate and even if MIP was successfully prepared, the predetermined specificity would lose its halo. Another reason for not being reported in the field of enantiomer separation is probably that MIP with small molecules like mimic enantiomers has already met the demand of chiral analysis. Despite of above reasons, application of protein MIP for enantiomer separation still needs to be discussed and studied, for it can efficiently avoid requirement of highly purified one enantiomer as template and can provide more active sites for chiral separation.

3.5. Partial filling technique

When sensing drawbacks of the use of proteins as chiral selectors that they are relatively expensive and present a strong absorbance in the UV region thus providing high detection limits in the determinations, several approaches were developed, among which the partial-filling technique was highlighted [136,137]. Using partial-filling as one way to immobilize proteins as chiral selectors has a parallel development with that of monolith, mainly because of its easy and historical applications, which can be reflected on several recent findings [138–141].

In the methodology, the capillary is partially filled with the chiral selector solution, leaving all the rest, including the detection window, free of the solution. The electrophoretic buffer is composed of plain buffer with no additive. The experimental conditions are selected so that the analytes migrate through the selector plug, where they have become separated, and are detected out of it [136]. In some cases, chiral selector plug length applied should be very large in order to obtain complete enantio-resolution of both analytes and strategies to either accelerate the elution of analytes or retard chiral selector plug should be applied in order to perform the analyte detection [137]. In order to slow the EOF without sacrificing other desirable analytical features such as short analysis time and resolution, a strict control and tuning of the interaction between buffer additives and capillary surface is needed.

Several authors have used partial-filling method for chiral selection or recognition. Hödl et al. [138] compared canine serum albumin and BSA for its chiral recognition ability using tryptophan derivatives as model compounds. It is shown that different tryptophan derivatives are dependent on the albumin species. Furthermore, the different functional groups of the derivatives show a marked influence on resolution. Gotti et al. [139] reported on the use of penicillin G acylase as chiral selector using a partial filling approach. The authors coated the capillary wall with pullulan for avoiding adsorption of the protein at the capillary wall and for EOF suppression. The coating was found to be unchanged after hundred injections. The system was tested using ketoprofen as a model compound.

Mart's group [140] deals with the application of the partial-filling technique for the separation of bupivacaine enantiomers by capillary electrophoresis using HSA as chiral selector. In this procedure the cationic surfactant cetyltrimethylammonium bromide (CTAB) was used as a dynamic capillary coating in order to reduce the electroosmotic flow and to detect both bupivacaine

enantiomers out of the chiral selector plug. The separated enantiomers out of the HSA plug (50 mbar 150 s) were detected in less than 4 min using 50 mM Tris, pH 8 as background electrolyte with 50 mM CTAB, at 30 °C and a separation voltage of 25 kV.

Another enantiomeric separation of promethazine and trimeprazine enantiomers, taking HSA as chiral selector, by affinity electrokinetic chromatography (AEKC)-partial filling is developed [141]. The estimated maximum and optimum resolution of trimeprazine and prometazine enantiomers (Rs = 1.74 and 2.01, respectively) corresponded to the following experimental conditions: [HSA] 170 μM and plug length 190 s at pH 7.5 and [HSA] 170 μM and plug length 170 s at pH 7.6 for trimeprazine and prometazine, respectively. The methodologies were applied for the enantiomeric quality control of promethazine and trimeprazine enantiomers in commercially available pharmaceutical formulations.

The enantiomeric resolution of compounds in partial filling technique is the result of a delicate balance between different experimental variables [142,143]. In order to select the experimental conditions for the enantio-resolution of five antihistamines, a multivariate optimization procedure was proposed [144,145] since univariate optimization can provide erroneous results when interactions between variables exist. This procedure includes the use of a Box–Behnken design for different experimental factors, such as running pH, HSA concentration and selector plug length and runs; pareto charts and partial least squares (PLS) analysis to study the significance of the main factors, as well as the corresponding cross-terms and squared effects; multiple linear regression to find the optimum experimental conditions [146].

3.6. Evaluation of monoliths

After fabrication of monolith (see Table 2), often a crucial step need to be undertaken, that is the evaluation process through various parameters from different perspectives. However, up to now, in contrast with traditional chromatographic columns, no strict or universal assessment system has been established. Paper between papers and column between columns, even if the same type, would appear totally in different types of measurements.

Several methods have been proposed to compare the performance of monolithic and packed columns, through the Hans Poppe plots [147], through various kinetic plots [148], and through column impedance [149], and of a few other approaches. The column impedance is a performance index that relates the hold-up time of a column and its efficiency [149], to the mobile phase viscosity and the inlet pressure available. The initial success of these various methods owes much to the early ignorance of the scientific community regarding the properties of monolithic columns and their advantages compared to those of packed columns. Their use has much contributed to clarify the situation.

Pore size distribution of monolithic columns has always been one of the most challenging issues and often characterized by a bimodal pore size distribution [150], throughpores controlling the column permeability, and mesopore structure controlling the mass transfer kinetics and column efficiency [151]. Porogenic distributions are often measured by mercury intrusion (MI), scanning electron microscopy (SEM), and nitrogen adsorption method, which have turned a routine test [152–156].

Protein content is of great importance in monoliths with protein as chiral selectors. Through infrared spectroscopy of bare monolithic material and monolith with immobilized chiral selectors, their comparison and adsorption strength can evaluate whether the successful immobilization is achieved or not. Another method to estimate protein content is to add some indicator. For example, an estimate of the total protein content in the monolith is obtained by injecting a 0.1% (w/v) solution of copper sulfate onto the AGP columns. This method uses copper sulfate as a probe for the overall ion-exchange capability of the immobilized proteins [157]. Using carbamazepine, an analyte that has a single primary site on AGP, to make frontal analysis can also estimate the binding capacity and total amount of protein in the AGP silica monolith.

4. Conclusion

Proteins as chiral selectors have gained wide popularity due to its high efficiency of enantioseparation, and monoliths, as a new generation packing material, bear the advantage of easy preparation and high permeability. Thus, application of monoliths for enantio-separation is fair and reasonable, which can conquer shortcomings such as high ultraviolet adsorption by proteins, protein losses and poor reproducibility. Recent advances in monolithic fabrication paved the way for more report on monoliths with protein functionalization, presenting a promising and alternative approach for enantiomer separations, but more exploration still needs to be done, like efficiency of immobilizing proteins, its affordability and disposability. Future works are expected to reduce the functionalized procedures, to prolong the material lifetime, as well as to increase the scope of applications in the pharmaceutical, environmental and diagnostic fields.

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