Korean J. Chem. Eng., **30**(7), 1448-1453 (2013)
DOI: 10.1007/s11814-013-0048-1

Enantioseparation of chiral ofloxacin using biomacromolecules

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(*Received 26 March 2013 • accepted 1 April 2013*)

Abstract−Natural biomacromolecules including bovine serum albumin (BSA), calf thymus DNA (ct-DNA) and fish sperm DNA (fs-DNA) were studied as the free chiral selectors to separate R- and S-ofloxacin enantiomers from racemic ofloxacin, combined with ultrafiltration and subsequent crystallization. First, the interactions between chiral ofloxacin and biomacromolecules including BSA, ct-DNA, and fs-DNA were investigated using circular dichroism and fluorescence spectroscopy. BSA exhibited stereoselective adsorption towards R-ofloxacin at pH 9.0 with an enantioselectivity of 1.23, while ct-DNA showed enantiospecific interaction with S-enantiomer with the selectivity of 1.70 at pH 5.0. One single-stage adsorption by BSA provides an enantiomeric excess in the permeate (e.e.$_p$) of 14% in S-enantiomer, and five-stage operations enhance the chiral resolution to reach the e.e.$_p$ value of 44%. R-enantiomer with an e.e.$_p$ of −26% can be obtained through one single-stage adsorption by using ct-DNA, and −85% can be reached by five-stage operations. Enantiomeric mixtures with the intial e.e. of 44% (S-) can be upgraded to 95% (S-) through subsequent crystallization. This programmable process of adsorption and desorption using BSA or ct-DNA as chiral selectors can be successfully applied to produce the enantiomers with highly optical purity.

Key words: Enantioseparation, Bovine Serum Albumin, DNA, Chiral Drug Ofloxacin

INTRODUCTION

Chirality, the most intriguing phenomenon in living organisms, has attracted increasing attention in the pharmaceutical industry, owing to great differences in pharmacological, toxicological and/or metabolic activities of enantiomeric drugs in living systems [1,2]. In 2010, seven of the top ten biggest-selling prescription drugs were comprised of single-enantiomers [3]. During crystallization more than 90% of crystalline racemates cannot form two individual enantiomeric crystalline phases [4]. To separate enantiomers, a variety of techniques involving high performance liquid chromatography and supercritical fluid chromatography [5], membrane-based separation [6,7], and chiral extraction [8,9] have been studied to separate enantiomers [10], using chiral selectors such as polysaccharide, cyclodextrin, macrocyclic antibiotic, crown ether, molecular imprinting polymer [11], etc. Biomacromolecules of proteins and nucleic acids, with the advantage of inherent chiral properties and no cellular toxicity, have been considered as promising alternatives to separate racemic mixtures [12-14]. Higuchi et al. separated racemic phenylalanine in a solution system containing bovine serum albumin or DNA, followed by ultrafiltration, and found that the concentration ratio of D-isomer to L-isomer in the permeate was susceptible to the additives including surfactant agents, lipid, and fatty acid [15, 16]. Later, Higuchi et al. prepared DNA-immobilized cellulose dialysis membranes to separate phenylalanine racemates and found that the pore size of the DNA-immobilized membranes was critical to affect the preferentially permeated enantiomer, i.e., D-phenylalanine permeated preferentially through DNA-immobilized membranes with a pore size <2.0 nm, whereas L-phenylalanine permeated preferentially with a membrane pore size >2.0 nm [17]. In addition, Iritani et al. studied the chiral separation of tryptophan racemates with BSA followed by ultrafiltration [18], while Chung et al. utilized human serum albumin (HSA) as a stereoselective ligand for tryptophan racemates through a polyethersulfone ultrafiltration membrane separation [12]. However, few reports have been found on the separation of chiral drugs using biomoleculues as the chiral selectors, except that Fu et al. prepared ibuprofen enantiomer with higher optical purity utilizing BSA-immobilized magnetic nanoparticles followed by subsequent crystallization [19]. Therefore, it is intriguing to explore a new combinatorial route to separate drug enantiomers in a solution system with biomacromolecules as the enantioselective ligands, combining with ultrafiltration or subsequent crystallization purification.

Pharmacological researches have shown that the antibacterial activity of the S-enantiomer (Fig. 1) of ofloxacin (levofloxacin, CAS Registry No. 100986-85-4) is 8-128 times higher than that of the R-enantiomer [20,21], while ofloxacin racemates do not exhibit enantiomeric phase separation in their crystals. We have developed an effective approach of enantiomeric enrichment of ofloxacin enantiomers using BSA and ct-DNA as the enantioselective ligands, respectively, combined with ultrafiltration and subsequent crystallization. First, the interactions between chiral ofloxacin and biomacromolecules including BSA, ct-DNA, and fs-DNA were investigated using circular dichroism and fluorescence spectroscopy. Next, multistage ultrafiltration was used to upgrade S- and R-ofloxacin. Five-stage adsorption by BSA gives an enantiomeric excess in the permeate (e.e.$_p$) of 44% in S-enantiomer. R-enantiomer with an e.e.$_p$ of −85% can be obtained through five-stage adsorption by ct-DNA. Finally, the enantiomeric mixtures at the starting e.e. of 44% (S) can be upgraded to 95% (S) through subsequent crystallization.

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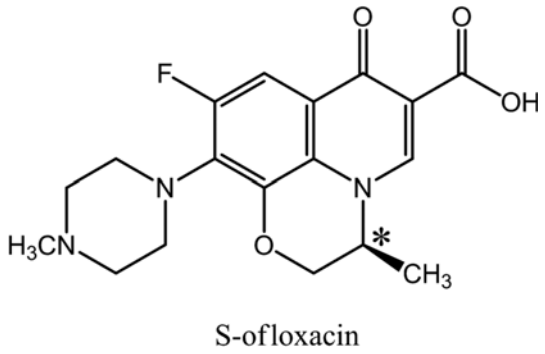

Fig. 1. Chemical structure of S-ofloxacin.

EXPERIMENTAL

1. Materials

Bovine serum albumin (BSA) (lyophilized powder, >97% purity), calf thymus deoxyribonucleic acid (ct-DNA) (white solid, >98% purity) and fish sperm deoxyribonucleic acid (fs-DNA) (white solid, >98% purity) were purchased from Sigma-Aldrich, USA. Racemic ofloxacin, and S-(-)-ofloxacin (levofloxacin or S-ofloxacin) (>99% purity) were purchased from Jianglai Biotechnology Corporation, Shanghai, China. Disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), sodium hydroxide (NaOH), phosphoric acid (H_3PO_4) and ethanol were purchased from Kermel Corporation, Tianjin, China. L-isoleucine and copper chloride were purchased from Guangfu Fine Chemical Research Institute, Tianjin, China. All the chemicals were analytical grade and used without further purification. Stirred ultrafiltration cell (model 8010) with a flat cellulose membrane (MWCL=10,000; d=25 mm) was purchased from Millipore Corporation, Germany.

2. Enrichment of Ofloxacin Enantiomers Using BSA or DNA

The typical operation for S-ofloxacin enrichment is as below. An ofloxacin racemate was added stirringly into 0.3 mM BSA phosphate buffer saline (PBS, 20 mM, pH 9.0) with the ofloxacin/BSA molar ratio of 1 : 3. After 15 min at room temperature, the mixture solution was separated using the ultrafiltration cell to provide the permeate, of which the concentration of individual ofloxacin enantiomer was measured by high performance liquid chromatography (HPLC) and the enantiomer excess value was denoted as $e.e._P$, calculated by Eq. (1).

$$e.e._P(\%)=\frac{(A_{S,P}-A_{R,P})}{(A_{S,P}+A_{R,P})}\times 100(\%) \quad (1)$$

where $A_{S,P}$ is the peak area of S-enantiomer and $A_{R,P}$ is the peak area of R-enantiomer in the permeate.

The stereoselectivity of biomolecules was denoted as α, calculated by Eq. (2).

$$\alpha=\frac{(C_{P,S}/C_{P,R})}{(C_{F,S}/C_{F,R})} \quad (2)$$

where $C_{P,S}$ and $C_{P,R}$ are the concentration of S- or R-enantiomer in the permeate, respectively. $C_{F,S}$ and $C_{F,R}$ are the concentration of S- or R-isomer in the feed solution.

On the other hand, the residue on the membrane was washed using equivolume phosphate buffer (pH 3.0) for 30 min, in order to make the BSA-adsorbed ofloxacin molecules desorbed. Then the washing solution was separated using the ultrafiltration cell again to provide the removal solution, of which the concentration of individual ofloxacin enantiomer was measured by HPLC and the enantiomer excess value in the residue was denoted as $e.e._R$.

$$e.e._R(\%)=\frac{(A_{S,R}-A_{R,R})}{(A_{S,R}+A_{R,R})}\times 100(\%) \quad (3)$$

where $A_{S,R}$ is the peak area of S-enantiomer and $A_{R,R}$ is the peak area of R-enantiomer in the residue.

R-ofloxacin enrichment is as below. Ofloxacin racemate was added stirringly into 0.6 mM ct-DNA base PBS (20 mM, pH 5.0) with the ofloxacin/base molar ratio of 1 : 6. Subsequent operations of adsorption or separation were similar to the procedures used in the enrichment of S-ofloxacin. The residue deposited on the membrane was washed using equivolume NaOH solution (0.1 M) for 30 min to make the DNA-adsorbed ofloxacin molecules desorbed.

The BSA chiral selectors were regenerated by incubation at 20 mM pH 9.0 PBS for 30 min, while ct-DNA selective ligands were regenerated by incubation at 20 mM pH 5.0 PBS for 30 min. Then, ultrafiltration processes were used to separate actified solution and regenerated biomolecules. Regenerated chiral selectors were reused for chiral separation of racemic ofloxacin. The transmembrane pressure was adopted as 0.25 MPa for each operation of ultrafiltration.

3. Purification of Ofloxacin Enantiomers through Crystallization

Crystallization purification was used to further purify the partially resolved ofloxacin solution. Enantiomeric mixtures with different initial e.e. (ee_0) value ranging from 44% to 83% were dissolved in 20 mL ethanol at 40 °C under stirring. After the sample was cooled at different cooling speed to crystallization temperature, the crystallization process was carried out at this temperature for 13 h. The crystals were separated from the solution by centrifugation at 8,000 rpm. The enantiomer excess of solid crystals and the supernatant was analyzed by high performance liquid chromatography (HPLC) separately.

4. Characterizations

The enantiomeric composition of the drug solution was measured by high performance liquid chromatography (HPLC, Agilent 1200) using a C18 stationary phase column (5 μm, 250×4.6 mm). The mobile phase was a mixture of methanol and water (20/80, v/v) containing 2.5 mM L-isoleucine and 0.6 mM cupric chloride. The flow rate was 0.5 mL/min and UV detection wavelength was set at 293 nm. Circular dichroism (CD) spectra were recorded with a Jasco J-815 spectropolarimeter equipped with a Julabo temperature controller. Each sample was measured at least three times in a 1 mm path length cuvette to get the average data. Fluorescence titrations were performed with a Cary Eclipse (Varian Ltd.). The excitation and emission wavelength was 350 nm and 370 nm respectively for ofloxacin. S- and racemic ofloxacin solutions (0.5 μM), respectively were titrated quantitatively with different molar ratio of ct-DNA/ofloxacin at 20 °C. Quenching constants of drug with DNA were determined by Eq. (4) reported in previous studies [22].

$$F_0/F=1+K_{SV}[Q] \quad (4)$$

where F_0 and F are the fluorescence intensities observed in the absence and presence, respectively, of quencher, [Q] is the quencher

concentration and K_{SV} is the Stern-Volmer quenching constant.

RESULTS AND DISCUSSION

1. Stereoselective Interactions between Chiral Ofloxacin and Biomacromolecules

BSA is the most abundant protein in blood plasma and has two typical binding sites for ligands: the warfarin-azapropazone site I (subdomain IIA) and the indol-benzodiazepine site II (subdomain IIIA) [23-27]. Previous reports on enantioselective interactions between chiral drugs and serum albumin by capillary electrophoresis have shown that ofloxacin was site I binding agents of BSA [28]. Here CD spectra were carried out to study the interactions of racemic ofloxacin or S-ofloxacin with BSA at pH ranging from 4.0 to 12.0. As shown in Fig. 2, addition of racemic ofloxacin induces small conformational changes to BSA at pH 9.0; however, it does not show any structural variation in the presence of S-ofloxacin under the same conditions. It is reasonable to conclude that BSA exhibits stereoselective interactions towards R-ofloxacin enantiomer.

Pharmacological research has shown that ofloxacin interacts directly with DNA in synergy with the gyrase enzyme [29,30]. Here, fluorescence titrations were performed to determine the binding affinities of S- or racemic ofloxacin to ct-DNA and fs-DNA, respectively. Fig. 3(a) shows that the fluorescence intensity of S-ofloxacin is gradually quenched as the concentration of ct-DNA increasing at pH 5.0. The quenching constants (K_{SV}) of S-ofloxacin and rac-ofloxacin with ct-DNA (Fig. 3(b)) are determined as 3.07×10^3 and 1.69×10^3, respectively, according to Eq. (4), suggesting stronger interactions between S-ofloxacin and ct-DNA than those with the racemate. In contrast, fluorescence spectra of S- and racemic ofloxacin show similar changes as titrated by fs-DNA, indicating that no stereoselective interactions exist between ofloxacin enantiomers and fs-DNA (Fig. S1).

2. Optimal Chiral Separation Conditions of Racemic Ofloxacin Using BSA and DNA

The influence of pH level and the chiral selector content on the chiral separation of ofloxacin was investigated by using BSA or DNA. As shown in Fig. 4(a), for 100 μM racemic ofloxacin experienced adsorption using 0.2 mM BSA selector; the $e.e._P$ value in the permeate reached the highest of 10% in S-ofloxacin at pH 9.0. While using 0.6 mM ct-DNA selector the highest $e.e._P$ value is −14% in R-ofloxacin at pH 5.0. In the case of fs-DNA no chiral separation efficiency is observed at pH ranging from 4.0 to 9.0, which is in accord with the above spectral analysis. Therefore, the optimal pH level is adopted as pH 9.0 for BSA and pH 5.0 for ct-DNA, respectively.

Fig. 4(b) shows the profiles of $e.e._P$ value against the concentration of chiral selectors at optimal pH values. In the case of using BSA chiral selector at pH 9.0, the $e.e._P$ increases with the BSA concentration (0.1-0.3 mM), and then remains around 17% at BSA concentration of 0.3-3 mM. Whereas, the absolute value of $e.e._P$ is en-

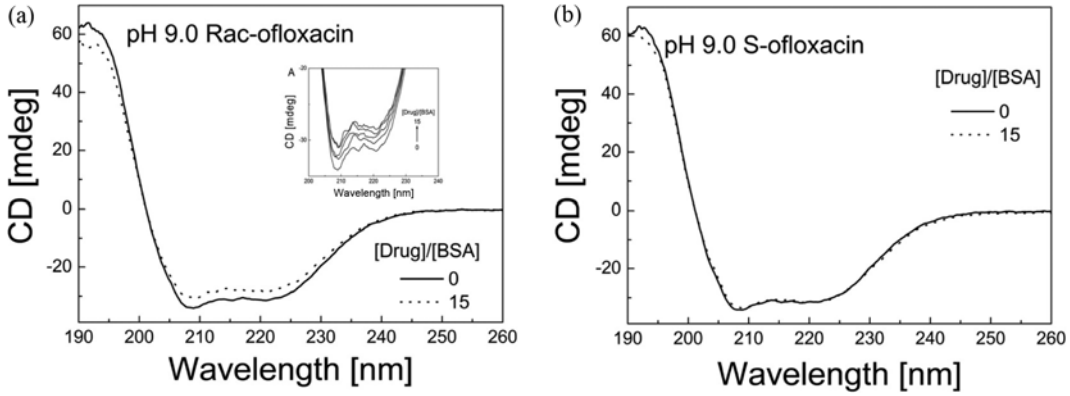

Fig. 2. CD spectra of BSA in the absence and presence of racemic ofloxacin (a) and S-ofloxacin (b) at the molar ratio of 15 at 25 °C. Insert: CD spectra of rac-ofloxacin titrations 3 μM BSA at the [drug]/BSA molar ratio of 0, 0.33, 5, 10, or 15.

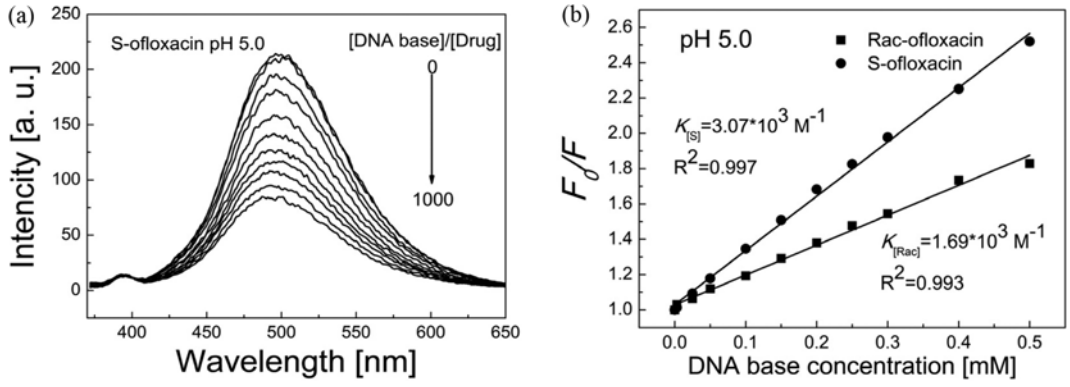

Fig. 3. (a) Fluorescence emission spectra of ct-DNA titrated with S-ofloxacin at different molar ratios (0, 6, 50, 100, 200, 300, 400, 500, 600, 800, 1,000); (b) Plot of F_0/F versus DNA base concentration for rac-ofloxacin and S-ofloxacin.

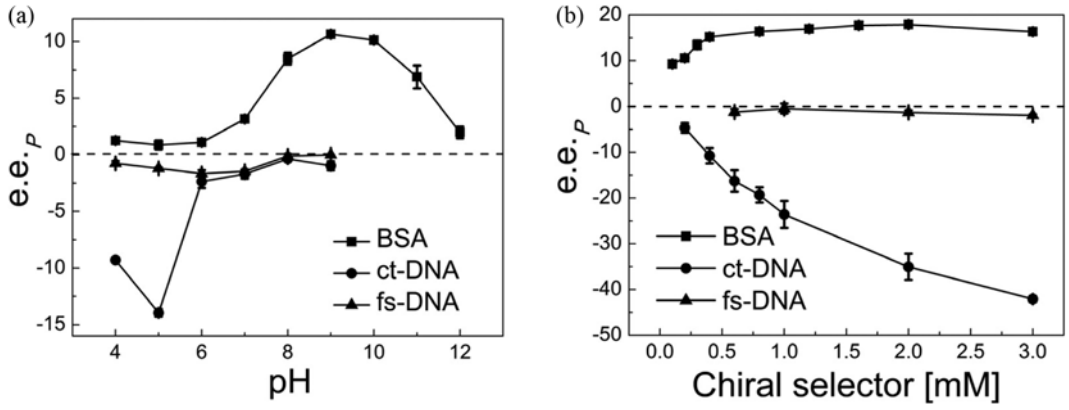

Fig. 4. (a) The e.e.$_P$ values of 100 μM racemic ofloxacin after adsorption by 0.2 mM BSA, 0.6 mM ct-DNA, and 0.6 mM fs-DNA at different pH levels; (b) The e.e.$_P$ values of 100 μM racemic ofloxacin after adsorption by different concentration of BSA at pH 9.0, ct-DNA and fs-DNA at pH 5.0 respectively.

Table 1. The e.e.$_P$ and e.e.$_R$ values of racemic ofloxacin separated by BSA[a] and ct-DNA[b]

Cycle	BSA		ct-DNA	
	e.e.$_P$	e.e.$_R$	e.e.$_P$	e.e.$_R$
1st	15.6	−10.3	−20.0	40.9
2nd	17.2	−14.2	−11.0	23.2
3rd	17.5	−13.2	−10.4	22.8

[a]1 mM BSA at pH 9.0

[b]0.8 mM ct-DNA at pH 5.0, 100 μM racemic ofloxacin

hanced greatly with the increase of ct-DNA concentrated from 0.2-3 mM, and reaches 40% after adsorption by 3 mM ct-DNA. As for using fs-DNA, no obvious change of e.e.$_P$ in the permeate is detected at the fs-DNA concentration of 0.5-3 mM. Therefore, BSA and ct-DNA were adopted to further study the regeneration and reusability of chiral selectors.

Table 1 lists the e.e.$_R$ value in the filtered residues using regenerated BSA and ct-DNA as the chiral selectors. Using BSA selectors, the e.e.$_R$ in the first cycle reaches −10.3% in R-enantiomer, while in the second and third cycle it is −14.2%. CD spectra of the BSA after desorbing the enantiomers at pH 3.0 exhibit a slight blue-shift (2 nm) for characteristic bands at both 222 nm and 208 nm. However, CD spectra of the BSA renatured at pH 9.0 for 30 min are identical to those of the native one (Fig. 5(a)). Adopting DNA selectors, the e.e.$_R$ is detected as 40.9% (S-excess) in the first cycle, then 23.2% and 22.8%, respectively, in the 2nd and 3rd cycles. As illustrated in Fig. 5(b), CD spectra of ct-DNA after desorbing the enantiomers at pH 13.0 show a blue-shift of 4 nm for characteristic band at 275 nm. Moreover, a slight red-shift of 2 nm occurs after the DNA is renatured at pH 5.0, which might contribute to the decreasing e.e.$_R$ in the 2nd cycle. Under optimized conditions, the enantioselectivity (α) of BSA towards R-ofloxacin is calculated as 1.23, while the α value of ct-DNA towards S-enantiomer is 1.70. Keurentjes et al. reported that about 190 theoretical stages are required for enantioseparation of chiral terbutalin to obtain 99% pure product when the enantioselective factor α=1.05, while the stage number can be decreased to 30 when α equals 1.20 for norephedrine enantiomers using liquid membrane with R,R- or S,S-di-hexyltartrate as the selector [31]. Therefore, with the corresponding enantioselective factors of 1.23 and 1.70, BSA and ct-DNA are promising chiral selectors to produce ofloxacin with highly optical purity.

As shown in Fig. 6(a), for the enantiomeric mixtures of rac-ofloxacin and S-ofloxacin, i.e., the mixtures with different initial ee$_0$ (enriched S-ofloxacin) of 0, 17%, 32%, 46%, 59% respectively, after experiencing a single-stage adsorption using BSA at pH 9.0, increased values Δe.e.$_P$ of 17.0%, 15.6%, 14.0%, 13.8% and 11.5% are obtained in the permeate. In addition, the enrichment of S-ofloxacin can also be achieved by using ct-DNA, as shown in Fig. 6(b). For the

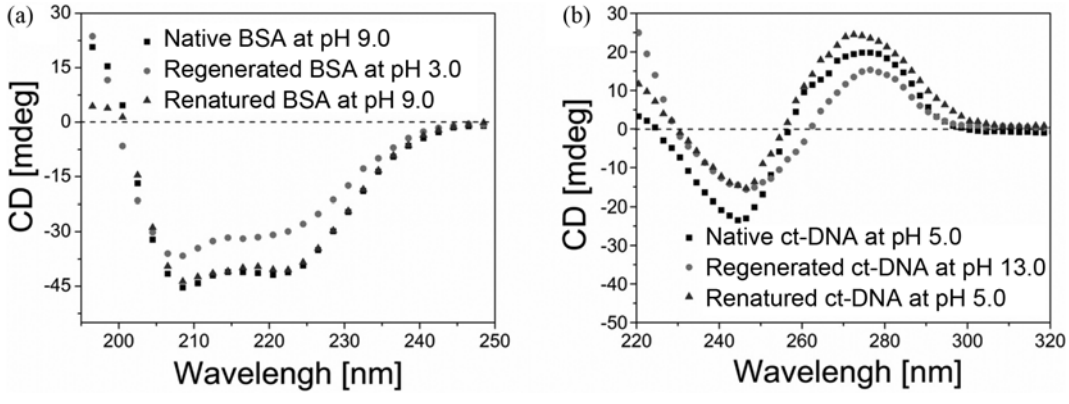

Fig. 5. CD spectra of native, regenerated, and renatured BSA (a) and ct-DNA (b).

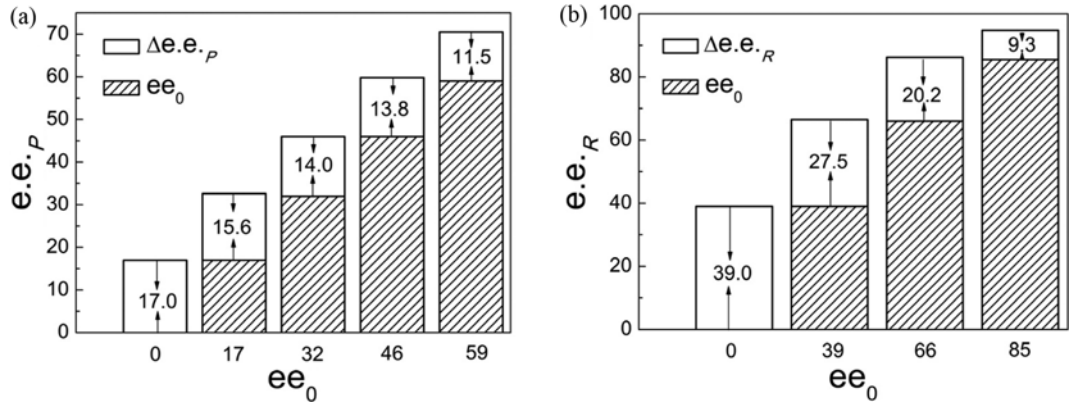

Fig. 6. (a) The e.e.$_P$ of 100 μM enantiomeric mixtures at different ee$_0$ (S-excess) after adsorption by 1 mM BSA; (b) The e.e.$_R$ of the desorbed enantiomers from 0.8 mM ct-DNA, which adsorbs the ofloxacin enantiomers in 100 μM mixtures at different ee$_0$.

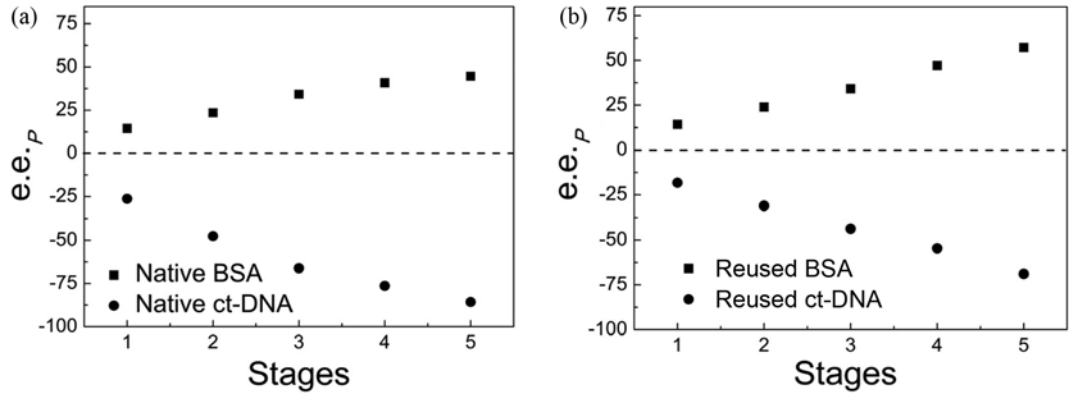

Fig. 7. The e.e.$_P$ of 100 μM racemic ofloxacin after multi-stage adsorption by native (a) or regenerated (b) 0.3 mM BSA and 0.6 mM DNA.

ee$_0$ of 0, 39%, 66%, and 85%, the Δe.e.$_R$ are determined as 39.0%, 27.5%, 20.2% and 9.3% respectively. Then, enantiomeric enrichment of S- or R-enantiomers was studied through the multi-stage adsorption using BSA and ct-DNA respectively. As shown in Fig. 7(a), the e.e.$_P$ are enhanced step-by-step with operational stages. After five stages of adsorption by using native BSA at every stage, the e.e.$_P$ value of 44% (enriched S-enantiomer) can be reached. In the case of R-enantiomer, the e.e.$_P$ of -85% (enriched R-enantiomer) can be achieved with five-stage adsorption by native ct-DNA. The related separation curves using HPLC are shown in Fig. S2. Moreover, a final purity of 50% (enriched S-ofloxacin) is obtained by using renatured BSA at every stage, while −73% of R-enantiomer is reached adopting renatured ct-DNA (Fig. 7(b)).

3. Upgrading e.e. of Partially Resolved Ofloxacin by Crystallization

To accomplish the efficient resolution of chiral drug with relatively low cost, we investigated a hybrid approach involving stereoselective adsorption and subsequent crystallization to upgrade e.e. of partially resolved mixtures of two enantiomers. We prepared the mixtures of ofloxacin enantiomers with different initial ee$_0$ of 44.5%, 55.6%, and 83.1%, respectively, and the e.e. value can be upgraded to 81.4%, 83.6% and 95.2%, respectively in the supernatant after single crystallization operation at the optimized conditions (Table 2). Meanwhile, the e.e. values are 26.9%, 32.5%, and 61.0%, respectively, in the solid crystals.

Table 2. The e.e. values of the supernatant and the precipitated crystals after crystallization

ee$_0$ (S-)	e.e. (S-)		Temperature/ °C	Cooling rate/ °C/min
	Supernatant	Crystal		
44.5	81.4	26.9	−10	0.5
55.6	83.6	32.5	−10	0.5
83.1	95.2	61.0	15	0.1

Several reports have shown that hybrid enantioseparation approaches combining enzyme kinetics [32], pertraction [33], membrane process with subsequent crystallization [34], can provide highly enantiopure products. The applicability of chiral resolution strategy involving stereoselective adsorption in an affinity ultrafiltration system and subsequent crystallization developed in this study is also relevant for other chiral selectors, and promising for enantioseparation of other chiral drugs.

CONCLUSION

We present the strategies for the enantiomeric enrichment of R- and S-enantiomers from racemic ofloxacin using natural biomacromolecules, including bovine serum albumin (BSA) and calf thymus DNA (ct-DNA) as free selectors, combined with ultrafiltration

and subsequent crystallization. First, the interactions between chiral ofloxacin and biomacromolecules including BSA, ct-DNA, and fs-DNA are investigated using circular dichroism and fluorescence spectroscopy. BSA exhibits stereoselective adsorption towards R-ofloxacin at pH 9.0 with an enantioselectivity of 1.23, while ct-DNA shows enantiospecific interaction with S-enantiomer with the selectivity of 1.70 at pH 5.0. Next, single-stage adsorption by BSA gives an enantiomeric excess in the permeate ($e.e._p$) of 14% in S-enantiomer, and five-stage operation enhances the chiral performance providing the $e.e._p$ value of 44%. R-enantiomer with an $e.e._p$ of −26% can be obtained through single-stage adsorption by ct-DNA, and this value increases to −85% by five-stage operation. Finally, the enantiomeric mixtures at the starting e.e. of 44% (S) can be upgraded to 95% (S) through subsequent crystallization.

ACKNOWLEDGEMENTS

This work was supported by NSFC (21076141, 21206107), and PCSIRT.

SUPPORTING INFORMATION

Additional information as noted in the text. This information is available via the Internet at http://www.springer.com/chemistry/journal/11814.

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Supporting Information

Enantioseparation of chiral ofloxacin using biomacromolecules

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(*Received 26 March 2013 • accepted 1 April 2013*)

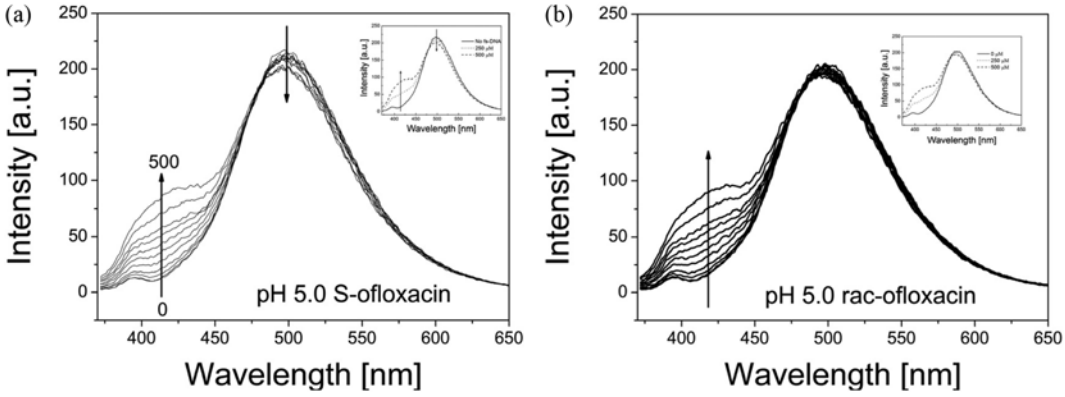

Fig. S1. Fluorescence emission spectra of S-ofloxacin (a) and rac-ofloxacin titrated with fs-DNA at different molar ratios (0, 6, 50, 100, 200, 300, 400, 500, 600, 800, 1,000). Insert: the typical fluorescence emission spectra at different concentration of fs-DNA base (0, 250 and 500 μM).

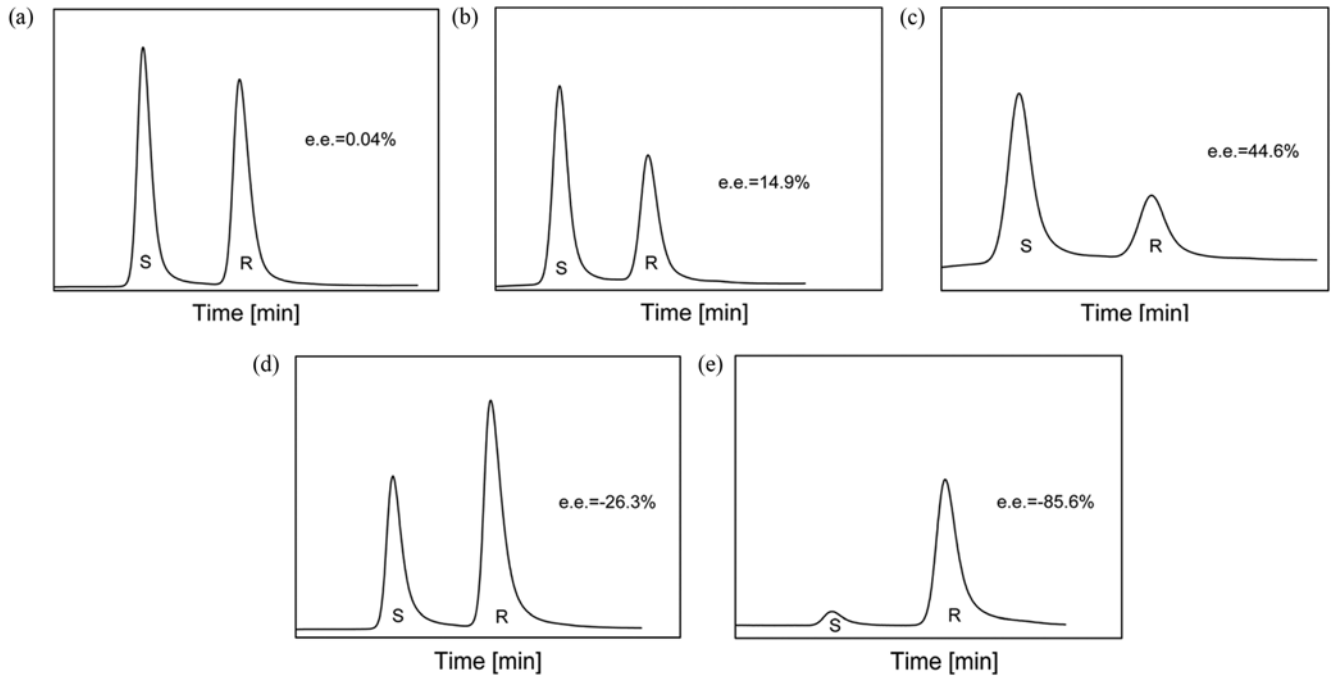

Fig. S2. HPLC chromatograms of 100 μM racemic ofloxacin (a), the resulting solutions of 100 μM racemic ofloxacin adsorbed by BSA with single (b) and (c) five stages at pH 9.0, as well as 100 μM racemic ofloxacin adsorbed by ct-DNA with single (d) and five (e) stages at pH 5.0.