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Enantioselective separation of chiral ofloxacin using functional Cu(II)-coordinated G-rich oligonucleotides†

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The DNA-based selector for discriminating chiral ofloxacin with high enantioselectivity and affinity is constructed through Cu(II)-coordination with G-rich duplex containing successive guanines. Using this chiral selector, *R*- and *S*-ofloxacin can be directly enriched from the racemate, with the enantiomeric excess of 85% (*R*) and 78% (*S*) individually by three operational stages.

Introduction

Along with the increasing demand of enantiopure drugs in the pharmaceutical industry, more attention has been paid to construct highly enantioselective selectors to discriminate left- and right-handed enantiomers. Chiral diversity is an intriguing nature of DNA macromolecules, which can serve as stereo-selective selectors for discriminating metallo-supramolecular complexes. The human telomeric antiparallel G-quadruplex can selectively interact with the P-Ni_2L_3, whereas the DNA three-way junction preferentially binds to the M-Fe_2L_3.[1] On the other hand, metals are in many respects the carriers of functions in biological systems, particularly, desired in the action of organic drugs targeting biomacromolecules.[2] Transition metals such as Cu^{2+}, Ni^{2+} or Pt^{2+}, preferentially coordinate at nitrogen N7 sites of guanines, consequently, they can anchor inside three-dimensional DNA structures to construct chiral microenvironments.[3] Previously using SELEX procedure the single-stranded DNA oligonucleotide with high enantioselectivity has been selected against a target enantiomer including oligopeptide, adenosin, tyrosinamide, amino acid derivatives, ibuprofen, and thalidomide derivative, showing attractive applications of DNA molecules for chiral resolution.[4]

The chiral drug ofloxacin, one of quinolone antibiotics which exhibit antibacterial activity by inhibiting the action of topoisomerase II, is originally proposed to bind to DNA.[5] The antibacterial activity of its *S*-enantiomer is 8–128 times higher than that of the *R*-enantiomer.[6] Since ofloxacin racemates do not exhibit enantiomeric phase separation in their crystals, it is a prerequisite to separate partially one enantiomer from the ofloxacin racemates through chiral resolving agents before crystallization. Herein we present a novel concept to construct a Cu(II)-coordinated double-stranded DNA-based selector with augmented enantioseparation efficiency, of which the core is the amplification of chiral recognition *via* the unique metal ion-coordinated DNA helix as well as the ligation between chiral drug and metal ion. Using this DNA-based selector, *R*- and *S*-enantiomer can be easily enriched individually from racemic ofloxacin, with the enantiomeric excess of 85% (*R*) and 78% (*S*), respectively, through three operational stages.

Experimental section

Materials

DNA oligonucleotides were purchased from the Japanese Takara Bio. (Dalian) with purity higher than 98% measured by HPLC. All DNA samples were annealed by heating the sample cuvette to 95 °C for 5 min in 10 mM Tris–HCl buffer (pH 7.0), followed by slowly cooling down to room temperature and storing at 4 °C. Polydeoxyguanylic–polydeoxycytidylic acid sodium salt (GC-DNA), polycytidylic acid–polyguanylic acid sodium salt (GC-RNA), calf thymus (ct-DNA, %GC = 42), micrococcus lysodeikticus (ml-DNA, %GC = 72), fish sperm (fs-DNA, %GC = 42) were purchased from Sigma-Aldrich (USA) with the purities higher than 98%. Bovine serum albumin (BSA, >97% purity) was also purchased from Sigma-Aldrich (USA). Racemate and *S*-enantiomer of ofloxacin with the purity of the mass fraction higher than 99% were purchased from Shanghai Jianglai Co., China. A Nanosep omega spin column (polyether salfone, 3k molecular weight cut off, PALL Life Sciences) was used to separate DNA–drug complex.

Enantioenrichment of *R*- and *S*-ofloxacin

For the G-rich oligonucleotides, concentrated racemic ofloxacin aqueous solution was added into the obtained 20 μM annealed DNA solution (strand concentration) in the absence or presence of Cu^{2+} ([Cu^{2+}]/[base] ratio of 0.1) at pH 7.0, to a final

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concentration of 0.02–0.2 mM. After 15 min incubation, the mixture was transferred to the spin column. After centrifugation at the speed of 8000 rpm for 20 min, the mixture solution was separated using the spin column to provide the permeate, and the DNA–Cu(II) complex with adsorbed ofloxacin enantiomers were trapped on the membrane. The concentration of individual ofloxacin enantiomer in the permeate was measured by high performance liquid chromatography (HPLC).

In order to remove the adsorbed ofloxacin enantiomers from the DNA sequences, an equivalent volume of Tris–HCl–EDTA buffer (pH 9.0) was added into the spin column to wash the trapped DNA on the membrane, and the mixture was incubated for 15 min. After centrifugation at the speed of 8000 rpm for 20 min, the mixture solution was separated using the spin column to provide the permeate, of which the individual concentration of the desorbed ofloxacin enantiomer from the filtered residue was measured by HPLC.

For the polynucleotides including fish sperm (fs-DNA), calf thymus (ct-DNA), micrococcus lysodeikticus (ml-DNA), polydG–polydC (GC-DNA), and polyG–polyC (GC-RNA), concentrated racemic ofloxacin aqueous solution was added into the obtained 800 μM DNA/RNA solution (base concentration) in the absence or presence of Cu^{2+} ([Cu^{2+}]/[base] ratio of 0.1) at pH 7.0, to a final concentration of 0.1 mM. The following operations on adsorption, desorption as well as separation were similar as the procedures used for oligonucleotides.

The adsorbed ofloxacin enantiomers were able to dissociate from DNA by using a desorption approach through both tuning pH and adding EDTA, therefore, the adsorption amount of individual *R*- or *S*-ofloxacin was calculated by eqn (1) and (2):

$$q_S = \frac{C_{S,\mathrm{R}} \times V}{C_{\mathrm{DNA}} \times V \times M_{\mathrm{DNA}}} \quad (1)$$

$$q_R = \frac{C_{R,\mathrm{R}} \times V}{C_{\mathrm{DNA}} \times V \times M_{\mathrm{DNA}}} \quad (2)$$

where $C_{S,\mathrm{R}}$ and $C_{R,\mathrm{R}}$ were the concentrations of *S*- and *R*-enantiomer in the filtered residue, respectively, C_{DNA} was the concentration of DNA, M_{DNA} was the molecular weight of DNA, and V was the volume of the separation system.

The enantioselectivity of DNA towards *S*-ofloxacin was denoted as α, calculated by eqn (3):

$$\alpha = \frac{(C_{S,\mathrm{R}}/C_{R,\mathrm{R}})}{(C_{S,\mathrm{F}}/C_{R,\mathrm{F}})} \quad (3)$$

where $C_{S,\mathrm{F}}$ and $C_{R,\mathrm{F}}$ were the concentration of *S*- and *R*-enantiomer in the feed solution.

For bovine serum albumin (BSA), concentrated racemic ofloxacin aqueous solution was added into 0.3 mM BSA solution at pH 9.0, to a final concentration of 0.1 mM. The ofloxacin enantiomers in the filtered residue were desorbed by adjusting the pH level to 3.0.

Multi-stage operation for enantioenrichment of individual *R*- or *S*-ofloxacin using G-rich oligonucleotides

In the enantiomeric enrichment of *R*-ofloxacin from the racemate, the *R*-enriched permeate obtained in the first operational stage was utilized as the feed solution in the next stage. Subsequent operations of adsorption or separation were similar as the procedures used in the single-stage operation. After three-stage adsorption, the enantiomeric excess in the permeate was denoted as $\mathrm{e.e._P}$ calculated by eqn (4):

$$\mathrm{e.e._P}(\%) = \frac{(A_{R,\mathrm{P}} - A_{S,\mathrm{P}})}{(A_{R,\mathrm{P}} + A_{S,\mathrm{P}})} \times 100(\%) \quad (4)$$

where $A_{S,\mathrm{P}}$ and $A_{R,\mathrm{P}}$ were the peak area of *S*- and *R*-enantiomer, respectively, in the permeate.

In the enantiomeric enrichment of *S*-ofloxacin from the racemate, the adsorbed ofloxacin enantiomers on the filtered residue in the first operational stage were desorbed using Tris–HCl–EDTA buffer (pH 9.0). Then the *S*-enriched desorption solution at the first stage was adjusted to pH 7.0 and utilized as the feed solution in the next stage. After three cycles of adsorption and desorption, the enantiomeric excess in the residue was denoted as $\mathrm{e.e._R}$ calculated by eqn (5), and the adsorption ratio of *S*-enantiomer was denoted as A_{S} calculated by eqn (6):

$$\mathrm{e.e._R}(\%) = \frac{(A_{S,\mathrm{R}} - A_{R,\mathrm{R}})}{(A_{S,\mathrm{R}} + A_{R,\mathrm{R}})} \times 100(\%) \quad (5)$$

$$A_S = \frac{A_{S,\mathrm{R}}}{A_{S,\mathrm{F}}} \times 100(\%) \quad (6)$$

where $A_{S,\mathrm{R}}$ and $A_{R,\mathrm{R}}$ were the peak area of *S*- and *R*-enantiomer, respectively, in the residue, $A_{S,\mathrm{F}}$ was the peak area of *S*-enantiomer in the racemic feed solution.

Characterizations

High performance liquid chromatography (HPLC). The enantiomeric excess of mixtures of ofloxacin enantiomers was analyzed by HPLC (Agilent 1200 Series). Chromatographic separations were performed using a Kromasil C_{18} 5 μm (4.6 × 250 mm) column, and detected by a UV detector at 293 nm using a mobile phase consisting of a mixture of methanol and water (20 : 80, v/v), 2.5 mM L-isoleucine, 0.6 mM Cu^{2+} at a flow rate of 0.5 mL min^{-1}. The injected sample volume was 20 μL.

Circular dichroism spectroscopy (CD). CD spectroscopy were carried out on a Jasco J-810 spectropolarimeter at 20 °C using a quartz glass cuvette with 0.1 cm path length. All the CD spectra were measured from 350 nm to 190 nm with a scanning speed of 100 nm min^{-1}. The cell holding chamber was flushed with a constant stream of dry nitrogen gas to avoid water condensation on the cell exterior.

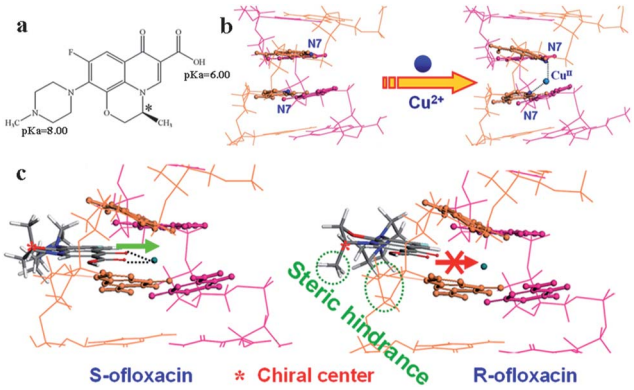
Isothermal titration calorimetry (ITC). ITC measurements were performed using a VP-isothermal titration calorimeter (Microcal, Northampton, MA). Titration was carried out by injecting 10 μL aliquots per injection of the concentrated *S*- or racemic ofloxacin solution into 5 μM DNA solution at 210 s intervals at 20 °C for a total of 28 injections. Titration curves were corrected for heat of dilution by injecting the ofloxacin solution into the buffer. The binding constant K_{A} and the enthalpy change ΔH^0 were obtained from the sigmoidal curve fitting.

Native polyacrylamide gel electrophoresis (PAGE). PAGE measurements were carried out to characterize the electrophoresis mobility of DNA structures using 20% acrylamide at 10 V cm^{-1} and 4 °C. The gel was stained in a 3 μg mL^{-1} Gelred solution for 30 min. PAGE images were obtained using a GDS8000 system (UVP, Inc., USA).

Results and discussion

In this study, double-stranded oligonucleotides with four tracts of successive GC pairs, including RET, c-kit2, and VEGF enriched in transcription initiation sites of proto-oncogenes, are selected as the DNA scaffolds (Table S1†).[7] CD spectra of all the double-stranded oligonucleotides show a positive peak at 265 nm and a negative band around 240 nm at pH 7.0, which is an indication of duplex structure (Fig. S1a†).[8] Upon addition of Cu^{2+} ($[Cu^{2+}]/[base] = 0.1$), CD spectrum of double-stranded RET at pH 7.0 shows a little decrease in the positive peak at 265 nm, and PAGE image suggests that RET–Cu(II) complex adopts a duplex structure (Fig. S1b and S2†). Cu^{2+} has been reported to bind preferentially with the duplex containing successive guanines on the same strand through the interaction between Cu^{2+} with N7 of guanines. This binding model occurs at the low $[Cu^{2+}]/[base]$ ratio and stabilizes the G-rich duplex.[9] The characteristic band of RET–Cu(II) exhibits an obvious decrease at 265 nm upon addition of *S*-ofloxacin, suggesting that the binding of ofloxacin occurs *via* an adaptive conformational change of DNA–Cu(II) complex. The induced CD band at 287 nm corresponds well to the UV absorption band of ofloxacin, indicating that the bound ofloxacin molecules are arranged along the helical DNA scaffold. Interestingly, CD spectrum of RET–Cu(II)–*S*-ofloxacin exhibits great difference to that of the RET–Cu(II)–racemate, indicating the unique enantioselective recognition towards *S*-ofloxacin against *R*-ofloxacin.

The stronger exothermicity is detected in the complexation of RET–Cu(II) with *S*-ofloxacin compared to that with the racemate, suggesting that RET–Cu(II) preferentially binds to the *S*-enantiomer (Fig. S3a and S3b†). The association constant (K_A) of RET–Cu(II) is determined as 9.8×10^5 M^{-1} for *S*-ofloxacin, which is comparable to the binding affinity of the DNA aptamer (IBA4) towards *S*-ibuprofen (K_d = 1.5 μM).[4e] However, in the absence of Cu^{2+}, no detectable exothermicity occurs in the binding of *S*-ofloxacin to RET (Fig. S3c†). For the polynucleotides involving natural calf thymus DNA, synthetic poly $[d(G\text{-}C)_2]$ and poly$[d(A\text{-}T)_2]$, the association constant for the formation of the *S*-ofloxacin–DNA complex was determined as 1.4×10^3 M^{-1}, 1.4×10^3 M^{-1} and 1.0×10^3 M^{-1}, respectively.[10] It is suggested that the Cu(II)-anchored DNA double helix significantly enhances the binding affinity of ofloxacin molecule owing to the coordination of carboxylic and carbonyl groups of ofloxacin with Cu^{II}.[11] As illustrated in Scheme 1c, for *S*-ofloxacin, drug molecule partially intercalates into the adjacent GC pairs and Cu^{2+} acts as a bridge between the adjacent N7 sites of nucleic acids and the carboxylic and carbonyl groups of ofloxacin. In the case of *R*-ofloxacin, its protrusion into the minor groove of DNA is prohibited to some extent by the steric hindrance between the methyl group of ofloxacin and the phosphate backbone of nucleic acids. Therefore, stereoselective recognition is achieved *via* Cu^{II}-coordinated G-rich double helix so as to discriminate ofloxacin enantiomers.



Scheme 1 (a) Chemical structure of *S*-ofloxacin; (b) Cu^{2+} coordinates at N7 sites of two successive guanines in the G-rich strand; (c) schematic illustration of stereoselective recognition on G-rich double helix for discriminating ofloxacin enantiomers (DNA model is constructed based on the crystal structure of $d[G_4C_4]_2$, PDB 2ANA), Cu^{2+} acts as a bridge between the adjacent guanines of nucleic acids and the carboxylic and carbonyl groups of ofloxacin.

The ability to tune the chiral recognition of DNA–Cu(II) complex with external stimulus is considered to be a dominant driving force to implement the chiral resolution through a programmable adsorption–desorption process. For the

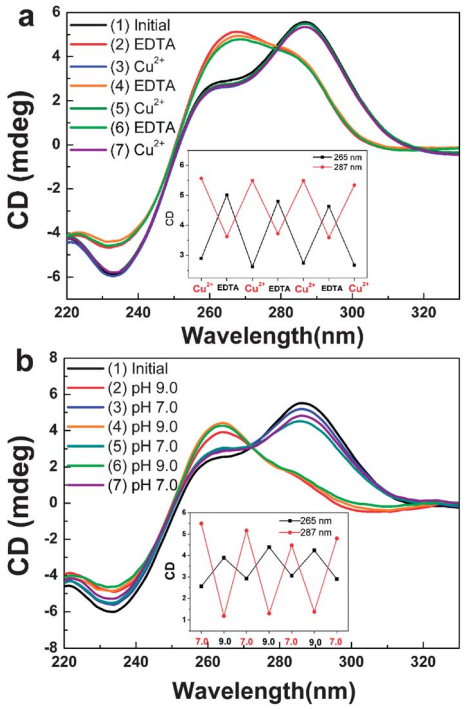


Fig. 1 (a) CD spectra of RET–Cu(II)–ofloxacin ($[Cu^{2+}]/[base] = 0.1$, 50 μM *S*-ofloxacin, pH 7.0) adding equiv. EDTA to Cu^{2+} alternately; (b) CD spectra of RET–Cu(II)–ofloxacin ($[Cu^{2+}]/[base] = 0.1$, 50 μM *S*-ofloxacin) at pH 7.0 and 9.0 reversibly by adjusting the pH through adding H^+/OH^-, respectively.

RET–Cu(II)–*S*-ofloxacin at pH 7.0, as shown in Fig. 1a, upon addition of equimolar EDTA to Cu^{2+}, the CD band shifts from 287 nm to 265 nm, suggesting that the strong chelation of Cu^{2+} induces the release of ofloxacin molecules from DNA. Moreover, the reversible CD spectra are recorded by adding EDTA and Cu^{2+} alternately. After removal of the bound Cu^{2+} and ofloxacin, RET restores its conformation of duplex (Fig. S4†).

As shown in Fig. 1b, the CD band shifts reversibly from 287 nm to 265 nm due to the pH level changing from 7.0 to 9.0 or *vice versa*, suggesting the pH-responsive recognition for drug enantiomers in the presence of Cu^{2+}. The weaker affinities between Cu^{2+} ions and DNA bases at basic conditions mainly contribute to the pH-dependent switches. Spectral reversibilities triggered by Cu^{2+} and pH value suggest that the supramolecular assembly of DNA–Cu(II)–ofloxacin is a highly dynamic structure controlled by reversible coordination bonding and electrostatic interactions, which are promising for the enantiomeric enrichment of either *R*- or *S*-enantiomer.

Cu(II)-coordinated DNA exhibits different adsorption behavior compared to that of DNA alone. Addition of Cu^{2+} ions obviously enhances the adsorption capacities of these three sequences for both *S*- and *R*-ofloxacin. Adopting the c-kit2-Cu(II), the adsorbed amount for *S*- and *R*-ofloxacin reaches to 0.21 and 0.092 mmol(ofloxacin)/g(DNA–Cu^{II}) respectively with the highest enantioselectivity (α) of 2.27 (Fig. 2a). Such adsorption capacities of DNA–Cu(II) are more than 100-fold higher compared to those of BSA molecules, the common selector for chiral ofloxacin (7.3×10^{-4} and 9.4×10^{-4} mmol(ofloxacin)/g(BSA) with an enantioselectivity of 1.28 towards *R*-enantiomer).[12] Meanwhile, the enantioselectivities (α) of either RET–Cu(II) or c-kit2–Cu(II) increase significantly in the range of 20–100 μM racemic ofloxacin in the feed solution, and the saturation can be reached at 150 μM racemate (Fig. 2b). The VEGF–Cu(II) complex shows larger adsorption capacity which is saturated with 200 μM racemate.

Highly efficient enantioseparation is usually performed in an operational mode of multi-stage adsorption. In the enantiomeric enrichment of *R*-ofloxacin from the racemate, after three-stage adsorption, the $e.e._P$ values can reach 74.8%, 85.1% and 75.0% for RET–Cu(II), c-kit2–Cu(II) and VEGF–Cu(II), respectively (Table 1). It is suggested that c-kit2–Cu(II) complex is the most efficient chiral selector for *R*-ofloxacin enrichment owing to the highest enantioselectivity. In contrast, adopting G-rich DNAs without addition of Cu^{2+}, the highest $e.e._P$ is only 22.0% for c-kit2 owing to lower adsorption capacity compared to that of the Cu(II)-coordinated DNA (Tables S2 and S3†). Although the similar enantioselectivities are detected for c-kit2 alone and c-kit2–Cu(II), significantly different efficiencies in the enrichment of *R*-enantiomer are obvious for these two adsorbents, which is attributed to the enhanced binding affinities of ofloxacin molecules to DNA through Cu(II)-mediated coordination.

In the enantiomeric enrichment of *S*-ofloxacin from the racemate, the DNA–Cu(II)-bound ofloxacin enantiomers on the filtered residue in the first operational stage are desorbed using Tris–HCl–EDTA buffer (pH 9.0). Then the *S*-enriched desorption solution is adjusted to pH 7.0 and then utilized as the feed solution in the next stage. After three cycles of adsorption and

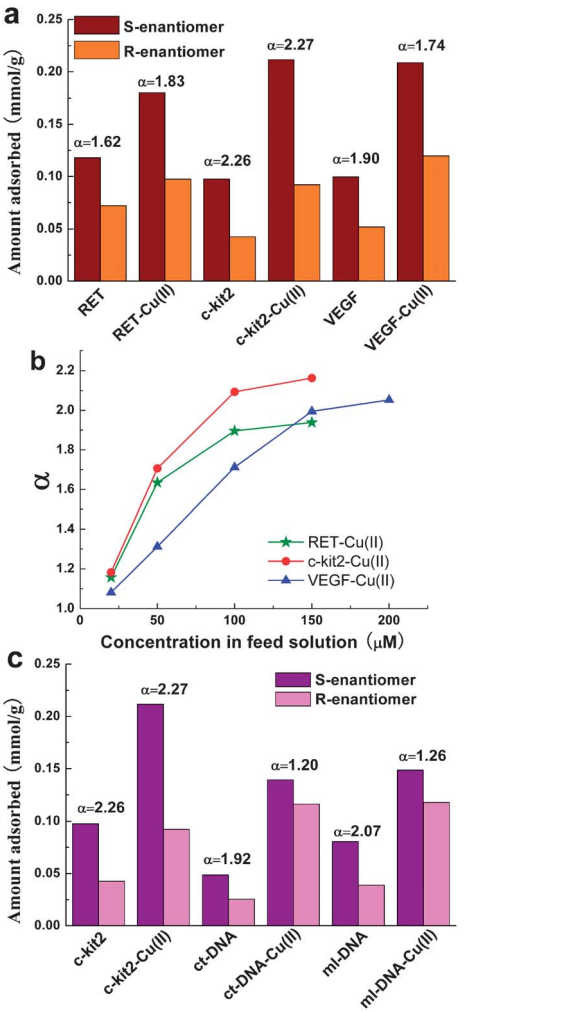


Fig. 2 (a) The adsorption amount of *S*- and *R*-ofloxacin individually as well as the enantioselectivities (α) towards *S*-enantiomer at 100 μM racemic feed solution, using 20 μM DNA–Cu(II) complexes ($[Cu^{2+}]$/[base] = 0.1) or 20 μM each oligonucleotide alone; (b) the enantioselectivities (α) towards *S*-enantiomer at different concentrations of racemic feed solution; (c) the adsorption amount of *S*- and *R*-ofloxacin individually as well as the enantioselectivities (α) towards *S*-enantiomer at 100 μM racemic feed solution, using DNA–Cu(II) complexes ($[Cu^{2+}]$/[base] = 0.1) or each polynucleotide alone.

Table 1 The $e.e._P$ in the permeate in the enrichment process of *R*-enantiomer and the $e.e._R$ in the residue in the enrichment process of *S*-enantiomer through three-stage adsorption using G-rich DNAs in the presence or absence of Cu^{2+a}

	$[Cu^{2+}]$/base = 0.1		Without Cu^{2+b}
Sequence	$e.e._P$ (%)	$e.e._R$ (%)	$e.e._P$ (%)
RET	74.8	49.5	21.0
c-kit2	85.1	78.1	22.0
VEGF	75.0	57.6	18.9

[a] Experimental conditions: 100 μM racemic feed solution, 20 μM DNA, pH 7.0. [b] The $e.e._R$ can not be determined since three-stage operation results in too low concentration.

desorption, the enantiomeric excess in the residue can reach 78.1% (*S*) using c-kit2–Cu(II) as the selector, with the yield of 30.3%. However, *S*-enantiomer can be hardly enriched through multi-stage operation using DNA without Cu(II)-coordination due to the poor adsorption capacity at low concentration of ofloxacin in the feed solution (Tables 1, S4 and S5†). It is indicated that amplification of both enantioselectivity and binding affinity of DNA selector is critical to reduce operational stages to obtain optically pure enantiomer. Therefore, Cu(II)-coordinated G-rich DNAs are promising chiral selectors to produce both *R*- and *S*-enantiomer from racemic ofloxacin with highly optical purity. Importantly, after three repetitious recycling, double-stranded c-kit2 maintains the adsorption capacity of *S*-enantiomer (only reduced by 8%) as well as the enantioselectivity (2.15), showing high efficiency in regeneration and reusability.

For comparison, several polynucleotides including natural fish sperm (fs-DNA), calf thymus (ct-DNA), micrococcus lysodeikticus (ml-DNA), and synthetic polydG–polydC (GC-DNA), polyG–polyC (GC-RNA), are also selected to perform the chiral resolution of ofloxacin enantiomers. As a result, adopting ct-, ml-, and GC-DNA, the α towards *S*-enantiomer is 1.92, 2.07 and 1.56, respectively, whereas no stereoselectivity is detectable for either fs-DNA or GC-RNA, indicating that the chiral recognition is greatly associated with DNA conformation (Fig. S1c†). Interestingly, addition of Cu^{2+} into ct-, ml-, or GC-DNA decreases the enantioselectivity significantly since polynucleotides are susceptible to undergo compaction process in the presence of transition metal ions.[13] For example, the α decreases from 2.07 to 1.26 while the adsorption capacities of both *S*- and *R*-ofloxacin exhibit obvious increments (Fig. 2c). Compared to other nucleic acid molecules, it is confirmed that the specific stereoselective selector with high enantioselectivity and affinity is constructed through unique Cu^{2+} coordination in the G-rich oligonucleotides for efficient enrichment of either *R*- or *S*-enantiomer.

Conclusions

The DNA-based selector for discriminating chiral ofloxacin with high enantioselectivity and affinity is constructed through Cu(II)-coordination with G-rich duplex containing successive guanines. Furthermore, Cu(II)-coordinated DNAs exhibit stimuli-responsive recognition towards ofloxacin enantiomers, providing a programmable adsorption and desorption process for the enantiomeric enrichment of either *R*- or *S*-enantiomer. Using this chiral selector, *R*- and *S*-ofloxacin can be directly enriched from the racemate, with the enantiomeric excess of 85% (*R*) and 78% (*S*) individually by three operational stages. Compared to other biomacromolecules, Cu(II)-coordinated G-rich DNAs are promising selectors for the enantioseparation of chiral ofloxacin.

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