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# Chiral magnetic microspheres purified by centrifugal field flow fractionation and microspheres magnetic chiral chromatography for benzoin racemate separation

**Ailin Tian**[1], **Jing Qi**[1], **Yating Liu**[1], **Fengkang Wang**[1], **Yoichiro Ito**[2], and **Yun Wei**[1,2,*]

[1]State Key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, 15 Beisanhuan East Road, Chaoyang District, Beijing 100029, P. R. China

[2]Laboratory of Bioseparation Technology, Biochemistry and Biophysics Center, NHLBI, National Institutes of Health, 10 Center Drive, Bldg. 10, Room 8N230, Bethesda, MD 20892, USA

## Abstract

Separation of enantiomers still remains a challenge due to their identical physical and chemical properties in a chiral environment, and the research on specific chiral selector along with separation techniques continues to be conducted to resolve individual enantiomers. In our laboratory the promising magnetic chiral microspheres $Fe_3O_4$@$SiO_2$@cellulose-2, 3-bis (3, 5-dimethylphenylcarbamate) have been developed to facilitate the resolution using both its magnetic property and chiral recognition ability. In our present studies this magnetic chiral selector was first purified by centrifuge field flow fractionation, and then used to separate benzoin racemate by a chromatographic method. Uniform-sized and masking-impurity-removed magnetic chiral selector was first obtained by field flow fractionation with ethanol through a spiral column mounted on the type-J planetary centrifuge, and using the purified magnetic chiral selector, the final chromatographic separation of benzoin racemate was successfully performed by eluting with ethanol through a coiled tube (wound around the cylindrical magnet to retain the magnetic chiral selector as a stationary phase) submerged in dry ice. In addition, an external magnetic field facilitates the recycling of the magnetic chiral selector.

### Keywords

Chiral magnetic microspheres; Centrifugal field flow fractionation; Microspheres magnetic chrial chromatography; Benzoin racemate

## 1. Introduction

The separation of enantiomers of chiral compounds continues to be of great interest due to their prevalence in the pharmaceutical industry, agrochemicals, and food additives to name a few. It is well established that enantiomers often exhibit different biological and pharmacological responses. Though high performance liquid chromatography (HPLC) and electrophoresis dominated chiral separation, and other various separation techniques were developed to solve this problem [1], separation of enantiomers still remains a challenge due to their identical physical and chemical properties in a chiral environment. And researches on specific chiral selectors along with the separation techniques continue to grow.

*Corresponding author: Yun Wei, State Key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, 15 Beisanhuan East Road, Chaoyang District, Beijing 100029, China, Tel & fax: 0086 10 64442928. weiyun@mail.buct.edu.cn.

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Magnetic nano-structured particles have been extensively exploited as the materials of choice for magnetic resonance imaging (MRI) [2], drug delivery [3], catalysis [4], as well as bioseparation [5] due to their strong magnetic response and low toxicity. The magnetic materials especially iron oxides carrying specific functional groups have also been applied for chiral discrimination. Those include carboxylmethyl-β-cyclodextrin-functionalized magnetic nanoparticles applied in the separation of chiral amino acids [6], capillary electrochromatography using β-cyclodextrin functionalized magnetic nanocomposites as tunable stationary phase [7], magnetic nanoparticles immobilized with chiral catalysts applied in asymmetric reactions [8], and BSA functionalized magnetic nanoparticles for the enantioselective resolution of chiral drugs [9].

By recognizing that magnetic cores covered with chiral ligands can possess both magnetic property and chiral recognition ability, magnetic chiral microspheres $Fe_3O_4$@$SiO_2$@ cellulose-2, 3-bis (3, 5-dimethylphenylcarbamate) (CMM) have been developed in our laboratory [10]. We found that the ligand cellulose-2, 3-bis (3, 5-dimethylphenylcarbamate) was suitable for the separation of a wide range of racemic compounds as described [11]. In addition, the use of $Fe_3O_4$@$SiO_2$@ cellulose-2, 3-bis (3, 5-dimethylphenylcarbamate) (CMM) has made the separation of racemate more efficient and easier. Significantly, an external magnetic field achieved almost complete and easy recovery of CMM from the solution to facilitate the recycling of CMM. However, there is still a drawback of low separation resolution of racemate as seen from the results of our previous work. The reason may be that the size of nano-materials is not uniform and/or active chiral center is masked by impurities. Size control of nano-materials is important to the discovery of intrinsic size/shape dependence [12]. Two general methods have been employed to create size-uniform nano-crystals. One method is direct particle sizing during synthesis by adjusting growth parameters [13–17]. The other is post-synthesis separation including filtration [18], electrophoresis, [19, 20] and chromatography [21, 22] that can produce particle fractions with narrow shape and size distributions. So this current study focuses on the development of a method to obtain uniform size chiral selector magnetic nano-materials free of impurities masking the chiral center, and then using this refined magnetic chiral selector the separation of racemate was performed through a long narrow coiled separation tube wound around a cylindrical magnet to retain the magnetic chiral selector as a stationary phase.

Field-flow fractionation (FFF) introduced by J. Calvin Giddings [23] is a separation technique where a field is applied to a particle suspension flowing through a long narrow channel, perpendicular to the direction of flow, which enables separation of the particles present in the fluid according to their differing movement under the force exerted by the field. In FFF, the field can be electrical, transverse flow through a semi-permeable membrane, centrifugal, magnetic, gravitational, thermal-gradient, etc. In the centrifugal FFF method applied in the present study, the separation mechanism is born from differences in particle movement under the applied force field where the parabolic laminar-flow-velocity profile in the channel determines the velocity of a particular particle according to its equilibrium position from the wall of the channel.

In this work, the uniform-size magnetic microspheres were obtained by the centrifugal FFF method for the first time, and then chiral separation was performed to resolve benzoin racemate with narrow tubing coiled around a cylindrical magnet, which was used to retain the magnetic chiral selector as a stationary phase.

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## 2. Materials and Methods

### 2.1. Reagents

1, 6-Diisocyanatohexane (DIH) was purchased from J&K. 3, 5- Dimethylphenyl isocyanate was obtained from Sigma (St. Louis, MO, USA) along with the racemic compound benzoin. All other chemicals including ferric chloride hexahydrate ($FeCl_3.6H_2O$), sodium acetate (NaAc), ethylene glycol (EG), ammonium hydroxide ($NH_3.H_2O$, 28wt%), tetraethyl orthosilicate (TEOS), microcrystalline cellulose (($(C_6H_{10}O_5)$n, n 40–400)), triphenylmethyl chloride and concentrated hydrochloric acid (HCl, 37.5%) were of analytical grade. All solvents used in the preparation process were of analytical reagent grade. Ethanol is purchased from Warner Graham Company (Cockeysville, MD, USA).

### 2.2. Chiral magnetic microspheres preparation

$Fe_3O_4$@$SiO_2$@CBDMPC (CMM) was prepared as described in our previous work [10]. Briefly, $Fe_3O_4$ magnetic microspheres were synthesized in ethylene glycol at 200°C for 8 h in a sealed autoclave. Then, the silica shell was coated onto the surface of $Fe_3O_4$ through a sol-gel approach to form core/shell-structured magnetic silica microspheres ($Fe_3O_4$@$SiO_2$). Finally, the chiral ligand cellulose-2, 3-bis (3, 5-dimethylphenylcarbamate) (CBDMPC) was fabricated to obtain chiral magnetic microspheres ($Fe_3O_4$@$SiO_2$@CBDMPC).

According to the previous result [10], the direct separation of chiral compound by the chiral magnetic microspheres ($Fe_3O_4$@$SiO_2$@CBDMPC) has not been successful. This may be caused by non-uniform size of chiral magnetic microspheres ($Fe_3O_4$@$SiO_2$@CBDMPC) and/or some unknown impurity masking the chiral center. Therefore, the uniform-sized chiral magnetic microspheres with active chiral center were obtained by the pre-treatment of CMM using the centrifugal field flow fractionation method as described below.

### 2.3. Centrifugal FFF pre-purification of chiral magnetic microspheres

The apparatus used was a type-J coil planet centrifuge purchased from P.C. Inc, Potomac, MD, USA. It is equipped with a separation column and a counterweight at symmetrical positions at a distance of 10 cm from the central axis of the centrifuge. In this centrifuge, the separation column revolves around the central axis of the centrifuge while it synchronously rotates about its own axis at the same direction. The separation column was made from a spiral tube support (made of Nylon by laser sintering for rapid prototyping) purchased from CC Biotech, Rockville, MD, USA. It has 4 spiral interwoven grooves, each 2.8 mm wide and ca 5 cm deep with 4 transfer radial grooves. The corner of the each spiral was rounded to prevent kinking of the tubing. The separation coil was made in our laboratory as follows: PTFE tubing of 1.6 mm ID (SW 14) (Zeus Industrial Products, Orangeburg, SC, USA) was flat-twisted and accommodated tightly by squashing it with a tool which fits to the radial grooves. The number of spiral layers is 10 and the total capacity is about 80 ml.

Pre-purification of crude CMM was performed as follows: The spiral column was first entirely filled with ethanol followed by injection of sample suspension (1 mL containing 30 mg of CMM) from the internal tail terminal of the spiral column. Then the column was eluted with ethanol at 8 mL/min, while the apparatus was rotated at 600 rpm (about 40 g force at the axis of the column holder). The effluent from the head end of the column was continuously monitored with a UV spectrophotometer (LKB Uvicord IIs, LKB Instruments, Stockholm, Sweden) at 280 nm and chromatogram was recorded using a strip-chart recorder (Millipore, Bedford, Boston, MA, USA). The fractions were collected into test tubes at 1 min/tube with a fraction collector (LKB Instrument). After the first peak was eluted, the column rotation was stopped while maintained at the same flow rate to collect the particles still retained in the column.

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### 2.4. Separation of benzoin racemate using chiral microspheres magnetic chromatography

The separation of benzoin racemate (Fig. 1) was performed by 0.85 mm ID, 3.8 m long PTFE tubing with a total capacity of 2.1 mL. The tubing was first filled with magnetic chiral selector particles (72 mg) suspended in ethanol, which was then wound around a cylindrical magnet to secure the retention of the chiral stationary phase. Then, 0.3 mL of sample solution containing 48 μg of benzoin racemate was injected and the column was eluted with ethanol at a flow rate of 0.1 mL/min. Since the effect of temperature on chiral separation is important and lower temperature will increase chiral recognition, the whole column was submerged in dry ice (−78.5°C) in order to improve the chiral selectivity. The effluent from the outlet of the column was continuously monitored with a UV monitor (Uvicord IIS) at 254 nm and fractionated into test tubes at 9 min/tube (0.9 mL/tube). The elution curve was drawn using a strip-chart recorder (Farmacia, Stockholm, Sweden) at a chart speed of 1 cm/20 min.

### 2.5. Analysis of magnetic microspheres

Fractions were analyzed by transmission electron microscopy (TEM) (Philip Tecnai 20, Netherlands) to confirm the size distribution and shape information. Dynamic light scattering (DLS) was conducted on Zatasizer Nano-ZS90 (Malvern Instruments, UK).

## 3. Results and Discussion

### 3.1. Pre-purification of CMM by centrifugal FFF

In most cases the separation method needs to be improved according to the microspheres' composition, surface property, average size, shape and size distribution as well as the matrix. Fig. 2 shows the separation of magnetic microspheres by centrifugal FFF. The chromatogram shows two peaks, the first small peak eluted at the void volume and the second major peak collected after stopping the column rotation. Without rotation, the particles were eluted near the void volume due to a lack of the centrifugal force.

From 30 mg of crude sample, an amount of 18 mg was obtained in the second peak. The results are quite reproducible. TEM was applied to image these fractions to qualitatively confirm the size distributions. The images are presented in Fig. 3, where fraction 1 contained the small size chiral microspheres with a mixture diameter of approximate 50 nm, 220 nm and fraction 2 contained the chiral microspheres with a geometrical diameter of approximate 240 nm. The TEM analysis results qualitatively confirmed the size distribution of microspheres is uniform after FFF separation. However, TEM need drying of the suspensions which could have induced additional aggregation of the CMM. DLS was also carried out and the result suggests that magnetic microspheres with a mean diameter of 240 ± 20nm and narrow size distribution (PDI=0.128) were obtained. The equilibrium velocity (V) of the magnetic microspheres suspended in a liquid in a given centrifugal force field (g) may be computed from the Stokes formula:

$$V=\frac{2r^2g(\rho_p-\rho_m)}{9\eta}$$

where r and $\rho_p$ indicate the radius and density of the particles; η and $\rho_m$ are viscosity and density of the suspending liquid, respectively. Inserting the actual value of these parameters in the present study (larger particles r = $1.20 \times 10^{-5}$ cm; $\rho_p$ = 1.6 g/cm$^3$; $\rho_m$ = 0.79 g/cm$^3$, η = 0.012 poise, g at the center of the column holder = $40 \times 980$ cm/sec$^2$ and that at the periphery of the spiral = $160 \times 980$ cm/sec$^2$), the equilibrium sedimentation velocities of 48 (minimum)–192 (maximum) μm/min were obtained. Since the channel in the column is less

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than 1.6 mm average ID (flat-twisted tubing) in diameter and ca 50 m in length (ca 80 mL capacity), the larger magnetic microspheres can be retained in the column at a flow rate of 8 mL/min due to the axial flow and strong centrifugal force at the periphery of the spiral channel, while smaller particles are eluted from the column because their sedimentation rate is too small. Under a high flow velocity, if the particles form aggregates, they will move faster than the average flow rate due to the axial flow effect, which might have happened in our samples. So the first peak included a few large particles. When the centrifugation is stopped, unit gravitational force cannot keep these larger particles in the column, since the diameter and relative density of the particles are too small, so that Stokes average sedimentation velocity becomes negligible.

### 3.2. Separation of benzoin racemate by magnetic chiral selector using microspheres magnetic chromatography

Using the purified magnetic chiral selector as a stationary phase, separation of benzoin racemate was performed by eluting ethanol through a long narrow coiled tube wound around a cylindrical magnet. As chiral recognition can be improved by lowering temperature [24], the whole column was submerged in dry ice during separation. Fig. 4 shows the chromatographic separation of D and L benzoin obtained at a flow rate of 0.1 mL/min at −78.5°C. D benzoin with no affinity to the chiral selector eluted immediately after the void volume (2 mL) while L benzoin, which has high affinity to the chiral selector, was substantially delayed in elution, resulting in complete separation of D and L benzoins (peak resolution is 1.5). The above separation performed under room temperature showed a single peak without separation due to reduced affinity of L-benzoin to the chiral selector. It should also be noted that the separation of the racemate with crude magnetic microspheres failed to resolve the D- and L-benzoin in dry ice, indicating that the pre-purification of the crude magnetic microspheres is essential for the successful separation of D, L- benzoin enantiomers. The chiral recognition of enantiomeric mixture of benzoin on CMM depends on the nature of the CMM, as illustrated in Fig. 5. The helical grooves provide a steric interaction site due to its highly organized structure. The residual carbamate groups offer both dipole-dipole and hydrogen bonding interaction sites. π-π interaction with aromatic species was served by the phenyl group. All these interaction sites of CMM contribute to the recognition process based on the three-point interaction mechanism [11, 25]. In addition, uniform size distribution and unmasked chiral center without impurity probably play an important role.

## 4. Conclusions

Using CMM with the uniform size distribution and free of impurity obtained by centrifugal field flow fractionation separation method, the benzoin racemate were successfully enantioseparated with peak resolution of 1.5. The best separation was achieved with 2.1 mL capacity column using ethanol at the flow rate of 0.1 mL/min under dry ice which increases the chiral selectivity. The chiral recognition of enantiomeric mixture of benzoin on CMM depends on the nature of the CMM, probably uniform size distribution and unmasked chiral center without impurity play an important role.

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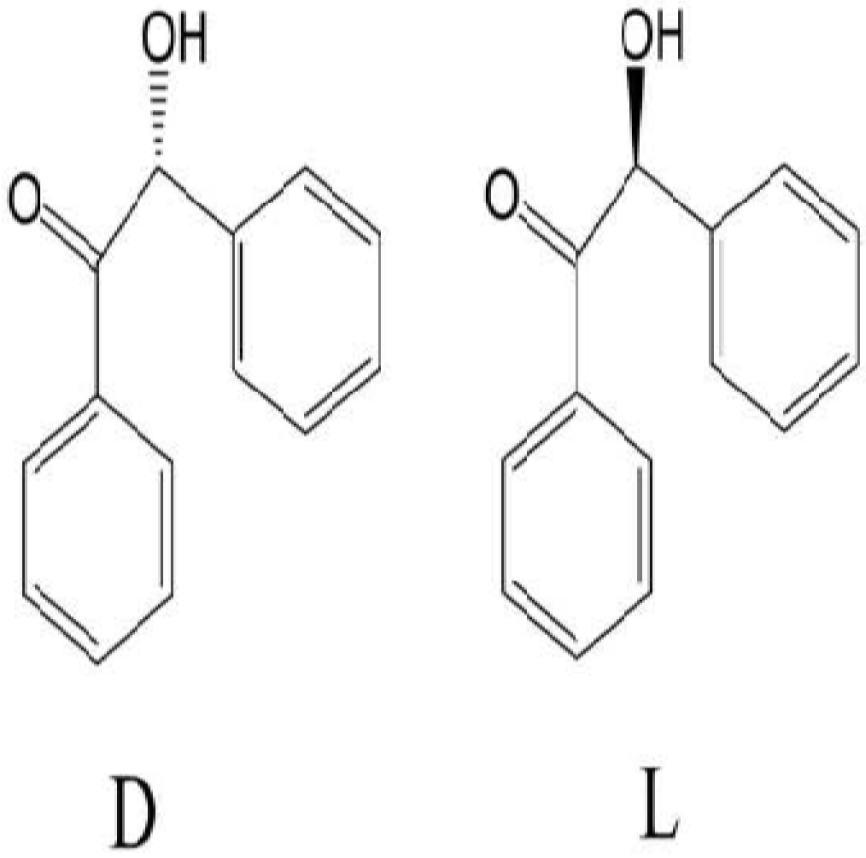


**Fig. 1.**
The structure of benzoin racemate

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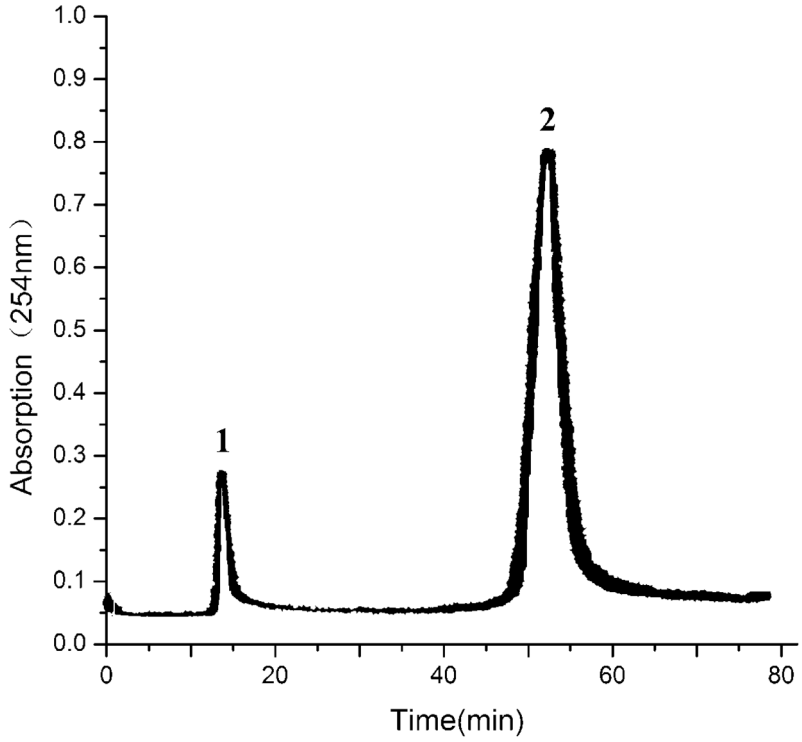


**Fig. 2.**
Centrifugal FFF separation chromatogram of chiral magnetic microspheres
Peak 1: First fraction of CMM
Peak 2: Second fraction of CMM

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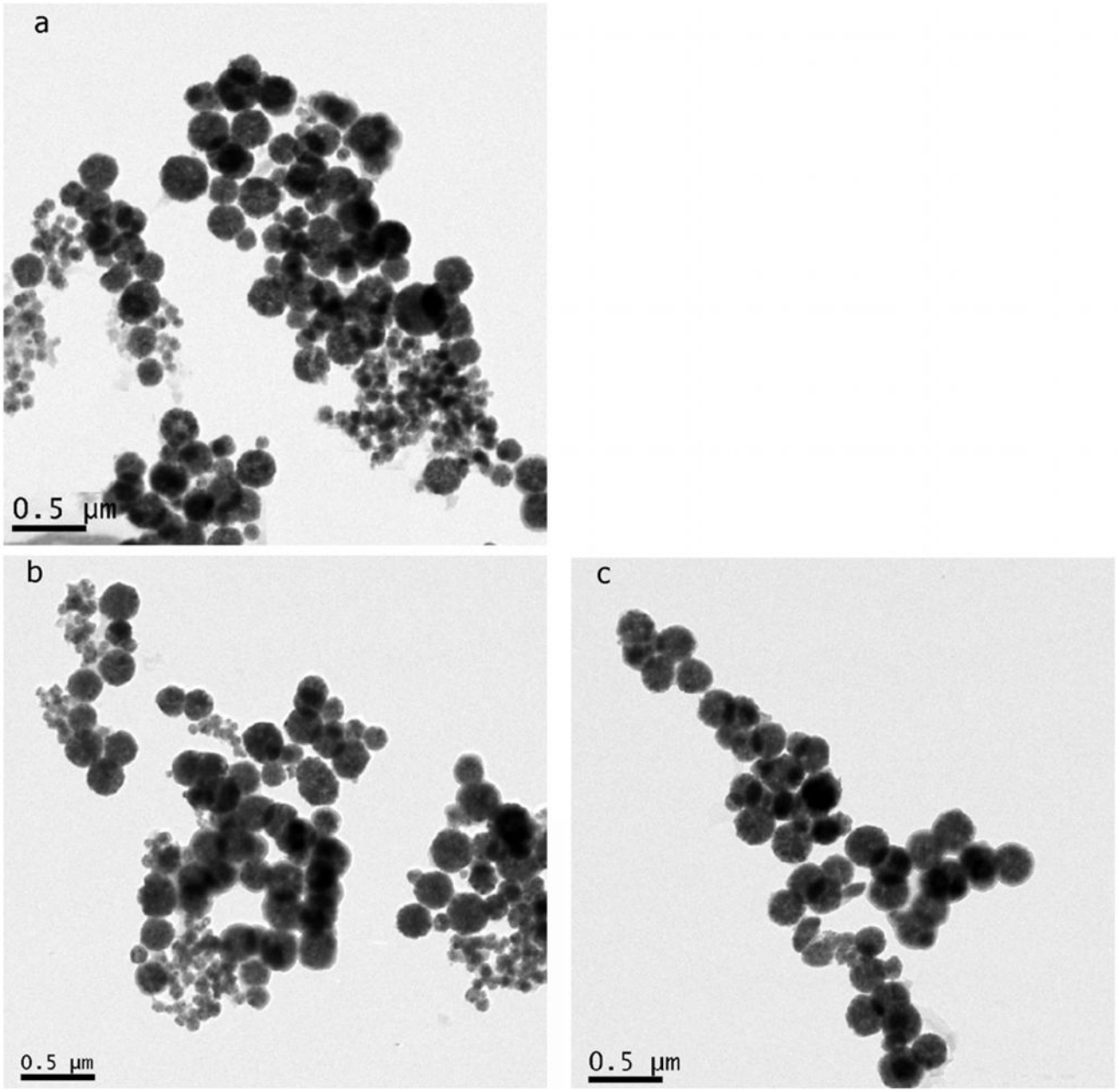


**Fig. 3.**
TEM of the CMM
a. Crude CMM
b. First fraction of CMM from FFF separation
c. Second fraction of CMM from FFF separation

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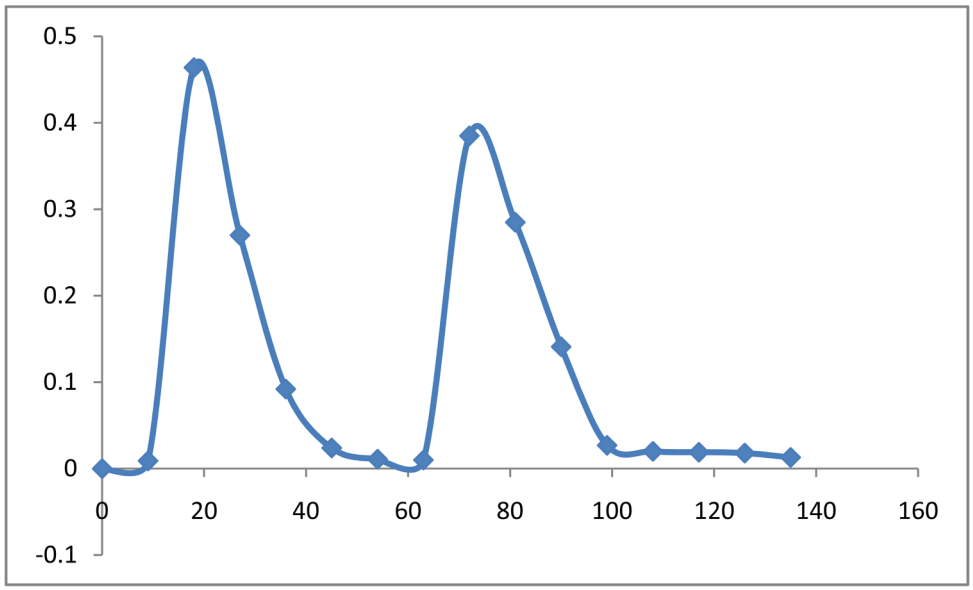


**Fig. 4.**
Chromatogram of Benzoin racemate after centrifugal FFF pre-purification of chiral magnetic microspheres

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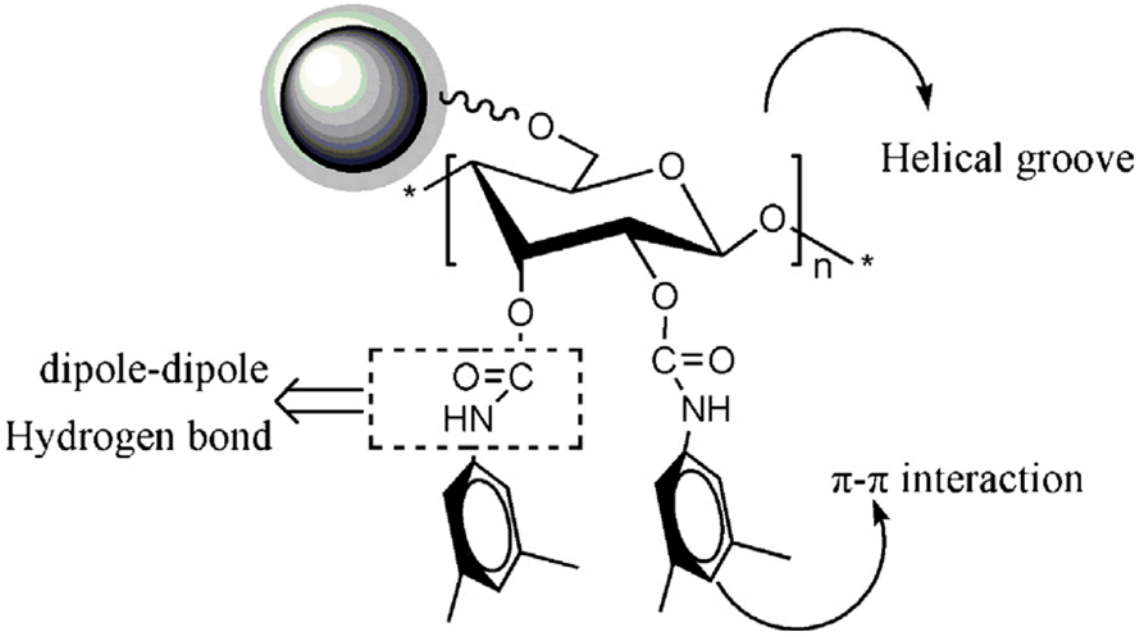


**Fig. 5.**
The chemical structure of CMM with its interaction sites