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ORIGINAL

Ligand Fishing with Cellular Membrane-Coated Magnetic Beads: A New Method for the Screening of Potentially Active Compounds from Natural Products

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Abstract Ligand fishing with targetbiomolecule-immobilized magnetic beads (MBs) has been established and developed for nearly 10 years. Advantages of this technique, such as the ease of operation, associated with a diversity of automated online approaches, make it a valuable tool for affinity studies. However, transmembrane proteins have not been used as the target biomolecules in the assay, since they are usually not available in a purified and bioactive form. In addition, few publications have reported the use of this method for screening active compounds derived from natural products. In this work, for the first time, cellular membrane-coated MBs, which to a large extent maintain the activity of the transmembrane proteins, were used for the fishing assay. We demonstrated application of red blood cell membrane-coated MBs for fishing potential active components from a natural product (*Angelica dahurica*). The potential active compounds, such as imperatorin, bergapten, and pabulenol, were detected. The result correlated well with cell membrane chromatography (CMC) coupled with HPLC. Comparisons of the developed MBs fishing assay with the CMC method showed the noteworthy advantages of the fishing technique regarding the consumption of cellular membranes, buffers as well as length of operation time.

Keywords Fishing · Magnetic beads · Red blood cell · Membrane · *Angelica dahurica*

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Introduction

Natural products are a main resource of drug candidates. For example, artemisinin, isolated from *Artemisia annua*, has been widely used in clinical studies as an effective antimalarial drug. Due to her remarkable contribution to the discovery of artemisinin, Tu Youyou won the 2015 Nobel Prize in medicine. Generally speaking, the screening of drug candidates from natural products is more time-consuming and more complicated than the other drug discovery methods. Numerous components should be initially isolated, and the biological activity of each of them determined. It is widely recognized that the screening process is an attractive and crucial starting point in drug development. However, the tedious screening procedures always decrease the efficiency and increase the cost of the drug development program [1]. Hence, methods embracing the ability to directly screen active compounds from natural products, thereby increasing the efficiency of this process, are tempting.

Since membrane proteins represent a prime target of over 50% of all modern pharmaceuticals [2, 3], cellular membrane affinity chromatography and cell membrane chromatography (CMC) were developed by Wainer et al. and He et al., respectively, to study membrane protein interactions and to screen bioactive compounds from complex mixtures [4–9]. In a recent review, Hou et al. [1] have shown that until May 2014, 23 CMC models have been used for the screening of active components from the traditional Chinese medicines

(TCMs). Though CMC is applicable and promising, complex procedures are usually needed for the preparation of the stationary phase and the packing of a chromatographic column. The latter process requires a high-pressure pump to pack the prepared stationary phase. This procedure is complicated and shows poor reproducibility, which may restrict the extensive applications of CMC [10].

Ligand fishing assay is an alternative method for the screening process [11]. Cell membranes (CMs) can be directly adapted for the fishing procedure. For example, Dong et al. used red blood cell (RBC) membranes for fishing potential active components from natural products. From the fishing eluent, four compounds were identified as potentially active by HPLC, MS, and NMR [12]. Though the method may be applied to predict the potential bioactivity of components in TCMs, it requires repeated ultracentrifugation steps to separate CMs from the active components before further analysis. The process is always time-consuming, and CMs not always completely separate from the active components and, therefore, interfere with further identification of the fishing eluent.

Ligand fishing assays were originally developed to extract proteins and enzymes from complex cell matrixes by immobilization of target biomolecules onto micro- or nano-sized beads [13]. Magnetic beads (MBs) are a kind of new material which could be used for active compounds. Recently, MBs have been used as the support for many assays [14, 15]. The manipulation of MBs is possible with the use of external magnets avoiding any contact with the analyte solution which prevents interference from the matrix (e.g., pH, ionic strength, or surface charges), during the magnetic extraction process. In 2007, Moaddel et al. [15] for the first time applied MBs in ligand fishing experiments. In their work, human serum albumin was immobilized onto the surface of silica MBs, to fish components from a mixture containing known ligands and non-ligands. The results correlated with data previously obtained with the use of bioaffinity chromatographic methods. Since then, a growing number of proteins have been immobilized onto MBs and used for fishing assays [14, 16–18, 20, 21]. The advantages of the method, such as the ease of operation associated with a diversity of automated online approaches, make the fishing technique a valuable tool for affinity studies. However, membrane proteins have never been used for this type of assay, and cell membrane-coated MBs have also never been used before for fishing ligands from natural products.

In this work, we describe for the first time the application of cellular membrane-coated MBs for fishing potentially active components from a natural product (*Angelica dahurica*). The active compounds such as imperatorin, bergapten, and pabulenol were fished out and characterized. The result correlated well with the CMC–HPLC results. Comparison between the developed MBs fishing assay and the CMC method in consumption of CMs, buffers, and operation time was also systematically studied.

Experimental

Chemicals and Materials

Magnetic beads with an average diameter of 1 μm were bought from Base Line Chrom. Tech. Research Centre (Tianjin, China). *A. dahurica* was obtained from LBX Pharmacy Co. (Tianjin, China). Acetonitrile, acetic acid, and fluorescein isothiocyanate isomer (FITC) were purchased from J&K Scientific Tech. Co. (Beijing, China). Verapamil was purchased from the National Institutes of Food and Drug Control of China (Beijing, China). Imperation was purchased from Yuanye Biotechnology Co. Ltd (Shanghai, China). Silica beads of 5 μm in diameter and 10 nm in average pore size were obtained from Nano-Micro Tech. Co. (Jiangsu, China). All the other chemicals were of analytical grade and obtained from local sources. BCA protein kit, used for quantification of cellular membrane proteins, was bought from Jiancheng Chemical Co. (Nanjing, China). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA).

Instrumentation

HPLC analyses were performed on an Agilent 1260 liquid chromatograph (Agilent Technologies, Palo Alto, CA). An Agilent Eclipse XDB C18 (4.6 × 250 mm, 5 μm) column was used. A UV-3100 UV spectrophotometer (Hitachi Co. Tokyo, Japan) was used to determine the protein concentration of the prepared CMs. A KQ-200VDE supersonic cleaner (Kunshan Supersonic Equipment Co., Jiangsu, China) was used for cell disruption. An IX71 fluorescent inverted microscope (Olympus, Tokyo, Japan) was used for imaging CMs, FITC-labeled CMs, MBs, and FITC-labeled CM-coated MBs. HPLC–MS was performed on Agilent 6210 ESITOF LC/MS system. CMC analyses were performed on an Agilent 1100 liquid instrument equipped with an Agilent ChemStation software (Agilent Technologies, Palo Alto, CA).

Extraction of Chemical Constitutes from *Angelica dahurica*

Ground powder (10 g) derived from crude herbs was extracted with 80 mL methanol for 30 min. The obtained extract was evaporated, and subsequently dissolved in 10 mL of deionized water. The obtained solution was filtered through a 0.22 μm membrane filter, and was stored at 4 °C.

Preparation of CM-Coated MBs

RBC CMs were prepared as previously described [4]. Briefly, blood was obtained from a rabbit, and was stored in an Alsever's solution at 4 °C until needed. The sample was centrifuged at 3000 rpm for 5 min and the obtained pellets were resuspended in a low osmotic solution. The suspension was sonicated for 10 min at 4 °C to disrupt the cells. The resulting homogenate was centrifuged at 200×*g* for 5 min and the obtained supernatant was further centrifuged at 15,000×*g* for 20 min at 4 °C. Thereafter, the supernatant was discarded and the pellet mixed with deionized water, from which the CMs' suspension was prepared. The membrane protein concentration in the suspension was determined by the BCA method. The obtained CMs' suspension was then stored at 4 °C until needed.

The process of coating CMs on MBs was expressed as an adsorption isotherm and measured as follows: 30 mg MBs were placed into a series of test tubes. Various concentrations of CM suspension (final volume 1 mL) were added to the tubes in a 5 mmol/L phosphate buffer (pH 7.4). The solutions in the test tubes were mixed in a shaker at 4 °C overnight. The concentration of CMs' proteins coated on MBs, in each tube, was determined by a BCA kit.

CMs' coating time was also evaluated. Briefly, 30 mg MBs were placed into a series of tubes and CMs' suspension was added to prepare CM-coated MBs (CMMBs). The concentration of CMs' proteins coated onto the MBs, in each tube was determined at 0, 2, 6, 10, and 24 h, respectively.

Preparation of FITC-Labeled CMs and FITC-Labeled CMMBs

The FITC-labeled CMs were prepared as described in our recent publication [19]. Briefly, a 1 mg/mL FITC solution was first prepared using DMSO as a solvent. The FITC solution (10 µL) was then slowly added to 1 mL of the CMs' suspension. The solution was protected from light and stored at 4 °C for 8 h. The obtained CMs were washed with 5 mmol/L PBS for a total of four times by centrifugation to remove any FITC in excess. The FITC-labeled CMs were immobilized onto the surface of MBs under vacuum and continuous agitation. The reaction mixture was subsequently stored at 4 °C and protected from light overnight. The mixture was washed with 5 mmol/L phosphate buffer (pH 7.4), until no residual CMs could be detected. The supernatant was then removed by centrifugation. The FITC-labeled CMMBs were imaged with a fluorescence confocal multiphoton microscope and CMs, FITC-labeled CMs, and bare MBs were also observed, for comparison.

Ligand Fishing by CMMBs

A mixture containing 100 µmol/L verapamil and 100 µmol/L thiourea was initially prepared to evaluate the selectivity of the assay. CMMBs were prepared as above and co-incubated with the mixture at 37 °C for 30 min. Then, the CMMBs were removed using magnetic force, and washed with 5 mM PBS, until no verapamil could be detected in the washing solution. Finally, the particles were vortex-mixed in 20% acetic acid. The obtained eluent was analyzed by HPLC method.

The stability of the ligand fishing method was evaluated by intra-day and day-to-day variation using verapamil as the sample. The CMMBs were prepared, and were subpacked into nine tubes, equally. All the tubes were stored at 4 °C. Among which, five tubes were evaluated in 1 day to obtain intra-day variation. The other tubes were evaluated day by day to obtain day-to-day variation. Ligand fishing from a natural source (*A. dahurica*) was also performed. Specifically, CMMBs were mixed with the extract of the *A. dahurica* in 5 mL tubes. The tubes were vortex-mixed thoroughly, and agitated for 30 min before the supernatant was removed. CMMBs were washed thrice with a 5 mmol/L phosphate buffer (pH 7.4), until the components of *A. dahurica* could not be detected in the washing solution. The obtained CMMBs were subsequently vortex-mixed in 20% acetic acid. The obtained solutions were lyophilizated and dissolved by acetonitrile before HPLC analysis. A control experiment was performed using 5 mmol/L PBS instead of *A. dahurica* extract. In addition, the fishing experiments were also performed with naked magnetic beads with the same procedures.

Active Compound Screening by CMC

RBC/CMC column (2 mm × 10 mm) was prepared according to our recent publication [19]. The column was equilibrated with 5 mmol/L phosphate buffer (pH 7.4), before it was used at 37 °C for CMC. *A. dahurica* extract was used as sample. The flow rate of the mobile phase (5 mmol/L phosphate buffer solution at pH 7.4) was set to 0.1 mL/min at 37 °C. The detection wavelength was 300 nm. The retained chromatographic peaks of CMC were carefully collected and further analyzed by HPLC.

Results and Discussion

Characterization of the CMMBs

Due to their easy-to-operate, MBs have been used for ligand fishing assays in several publications [16–18]. Since silica supports have been proven appropriate for adsorption of

CMs in CMC and micro-CMC techniques [4–10], silica-coated MBs were selected for the immobilization of CMs in this work. As shown in Fig. 1, CMs derived from rabbit RBCs were physically adsorbed onto the MBs (Fig. 1a). The prepared CM-coated MBs were used for ligand fishing, and the fishing process is shown in Fig. 1b.

Interactions between receptors located on CMs and their ligands were the basis of the fishing process. Washing step (Fig. 1b) is to get rid of the aspecific bindings and in the meantime to keep the specific binding between membrane receptors and their ligands. PBS is a solution with good biocompatibility. It has been used for washing out the aspecific bindings from RBC membranes [12]. Therefore, it was used as the washing solution in this work. The elution step (Fig. 1b) is to release the specific binding active compounds from receptors. In the previous study, many solutions were employed, such as 1% acetic acid solution, 10 mM ammonium acetate solution (pH 7.4) containing 20% (v/v) methanol or 50% (v/v) acetonitrile, 20% acetic acid solution, and so on [12, 16, 20, 21]. Among them, only 20% acetic acid solution was proven useful for eluting bound compounds from CMs [12]. In this solution, most of membrane receptors

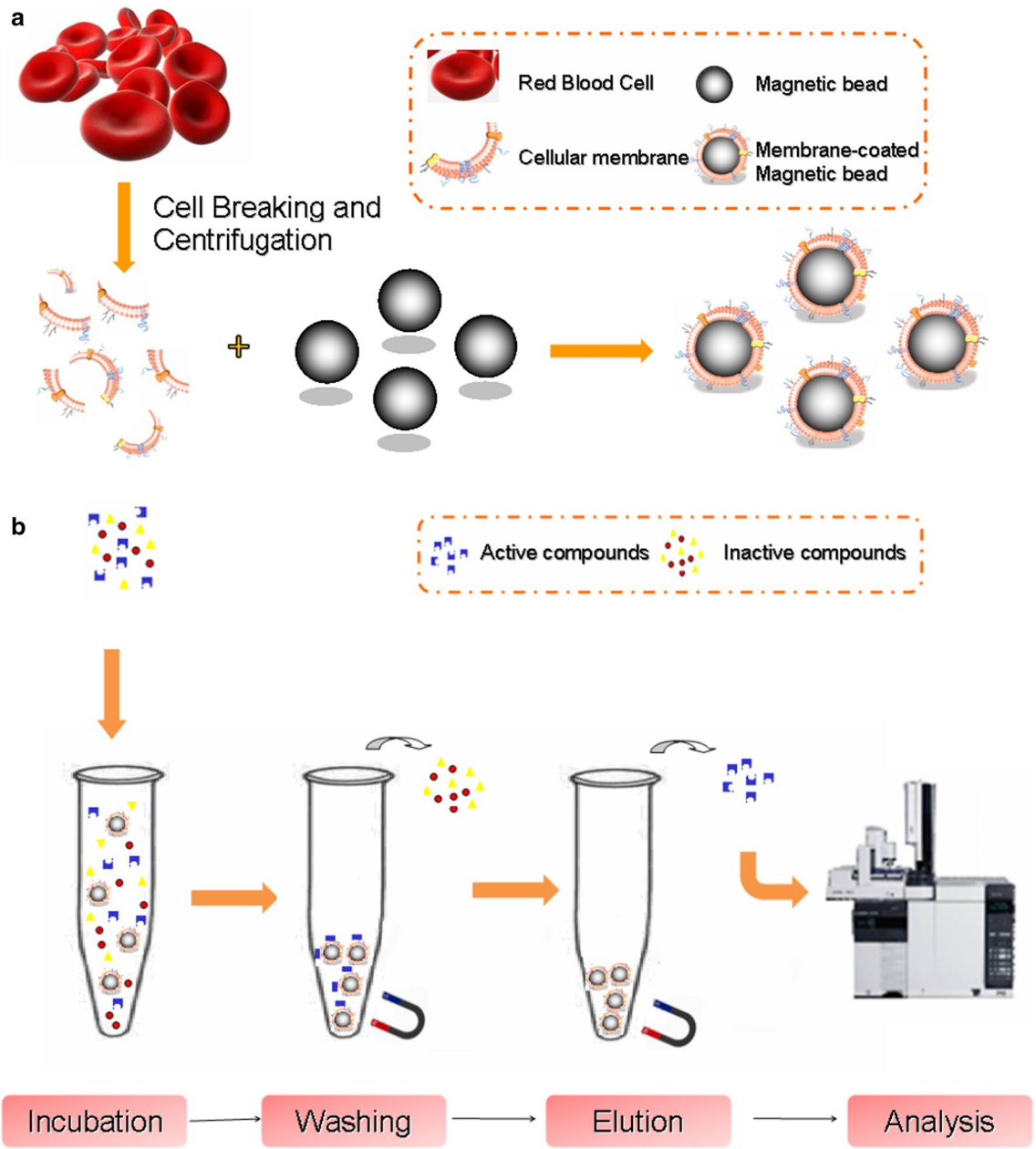

Fig. 1 Scheme of immobilization of cellular membranes (CMs) on magnetic beads (MBs) (**a**) and fishing process of active compounds by cellular membrane-coated magnetic beads (CMMBs) (**b**)

were denatured, therefore, releasing their ligands. The other solutions were only reported as eluents to release compounds from known protein receptors (such as HSA [21]). Different solutions are always needed to elute ligands from different receptors. Most of receptors on CMs are unknown, so the 20% acetic acid solution was used for this work.

Elution time from 30 min to 2 h was also characterized by determining verapamil concentration in the elution buffer with HPLC. As a result, no apparent difference of the content of verapamil was found. Therefore, 30 min was used for the elution time. The same elution time was also adopted by McFadden et al. to elute ligands from His6-tagged calmodulin-immobilized MBs [16].

The process of CMs' immobilization on MBs was also optimized. The maximum adsorption capacity of CMs was determined. Similar to previous studies [10], the amount of membrane proteins' concentration was adopted to express the amount of CMs. Figure 2a shows the adsorption isotherm curve of CMs' immobilization on MBs. CMs immobilized onto MBs (Cs) initially increased with the increase concentration of CMs added to the immobilization reaction (Cm). When Cm was higher than 1.2 mg/mL, no apparent increase of Cs was observed even with increasing values

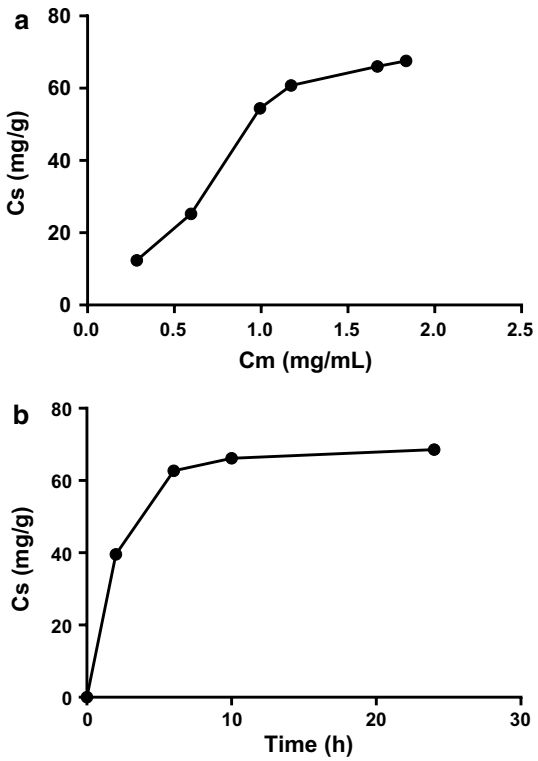

Fig. 2 Adsorption isotherm of red blood cell (RBC) membranes on the surface of magnetic beads (MBs) (**a**). Effect of incubation time on adsorption capacity of RBC membranes of MBs (**b**) (*Cm* is protein concentration of CMs, and *Cs* is the amount of milligram of CMs' protein per gram of MBs)

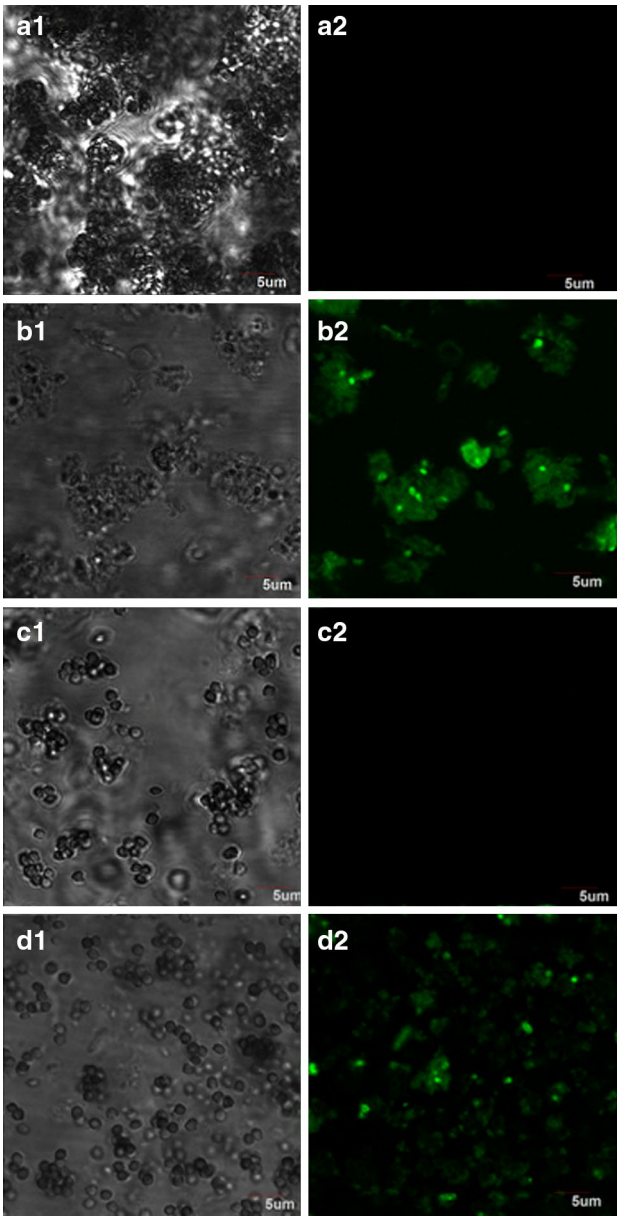

Fig. 3 Transmitted light images of RBC CMs (**a1**), FITC-labeled CMs (**b1**), FITC-labeled bare MBs (**c1**), and FITC-labeled CMMBs (**d1**). Fluorescent images of the prepared CMs (**a2**), FITC-labeled CMs (**b2**), FITC-labeled bare MBs (**c2**), and FITC-labeled CMMBs (**d2**)

of Cm. The reaction time for the immobilization process was also characterized. As shown in Fig. 2b, Cs significantly increased with the reaction time (up to about 6 h), and then reached plateau. Similar results were also reported in the other publications describing the immobilization of CMs on silica beads [4]. According to the results shown in Fig. 2, Cm of 1.2 mg/mL with a Cs value of 60.8 mg/g and a reaction time of 6 h were employed for the preparation of CMMBs used in this work.

To further confirm the CMs' immobilization on MBs, FITC-labeled CMs were immobilized onto the MBs and imaged by a fluorescence confocal microscope. After labeled by FITC, CMs did not show obvious structural change (Fig. 3a1, b1) and were able to emit green fluorescence (Fig. 3a2, b2). The MBs used in this work displayed a good sphericity with a diameter of about 1 μm (Fig. 3c1) and were unable to emit fluorescence (Fig. 3c2). FITC-labeled CMs were immobilized onto MBs and the resulting FITC-labeled CMMBs also displayed a clear spherical structure (Fig. 3d1). Importantly, the CMMBs were clearly visualized by fluorescence microscopy (Fig. 3d2), further supporting the successful immobilization of CMs onto MBs.

Selectivity and Stability of the CMMBs' Fishing Process

Calcium channel proteins, laid on the cellular membrane of RBC, could interact with calcium antagonists such as dihydropyridine drugs. Upon the immobilization of RBC membranes, the prepared CMMBs could be used for selective fishing of CM-interactive compound. Therefore, a mixture of verapamil (a widely used dihydropyridine drug) and thiourea (as a negative control) were selected as sample for characterization of the selectivity of the fishing assay [4, 22]. The result is shown in Fig. 4. Both verapamil and thiourea were detected in their mixture (Fig. 4a). After the mixture was incubated with CMMBs, only the active compound (verapamil) was found in the chromatogram of the elution (Fig. 4b). It suggests that the prepared CMMBs can selectively bind verapamil (due to its interaction with calcium channel proteins immobilized on the CMMBs). To further verify the specific interaction with the CMMBs, bare magnetic beads were also used for performing the fishing experiments (Fig. 4c). As can be seen in Fig. 4c, both verapamil and thiourea were not found in the eluates from bare magnetic beads. Besides, no apparent compound was detected in the solution obtained by directly analyzing the elution of the CMMBs without incubation with the sample (Fig. 4d). Therefore, the results shown in Fig. 4 indicate that CMMBs can be used for selective fishing of compounds that specific interact with cellular membranes from a mixture.

The repeatability of the CMMBs' fishing process was evaluated using verapamil as the sample. As a result, inter-day relative standard derivation (RSD) of 5.4% ($n = 5$) was obtained, suggesting good inter-day repeatability. Day-to-day RSD of 15.9% ($n = 3$) was obtained. Gradual decrease of the verapamil content in the elution solution was found. It suggests that biological activities of CMMBs decrease day by day. Therefore, freshly prepared CMMBs should be employed in this fishing assay to avoid the gradually decrease of activity.

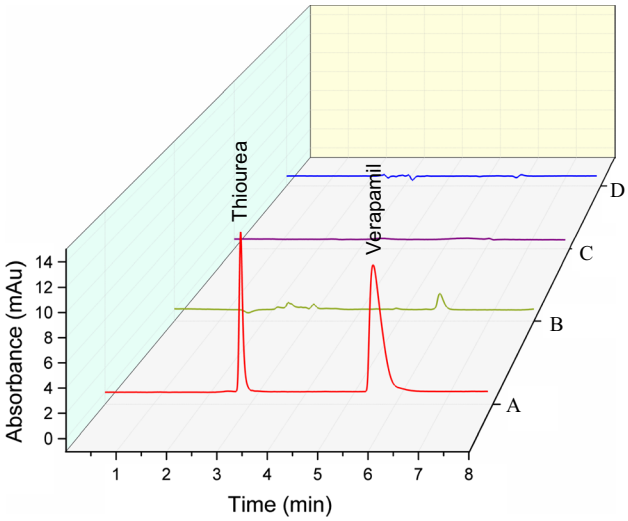

Fig. 4 HPLC results of mixture of verapamil and thiourea (**a**), the elution of CMMBs following incubation with the verapamil and thiourea mixture (**b**), the elution of bare MBs after incubation with the verapamil and thiourea mixture (**c**), and the elution of CMMBs without incubation with any solution (**d**). *Column* Agilent Eclipse XDB C18 (4.6 × 250 mm, 5 μm); Column temperature: 30 °C; detective wavelength: 265 nm; mobile phase: acetonitrile: 0.1% formic acid–water solution = 40:60; and flow rate: 0.8 mL/min

Fishing capacity of CMMBs was also determined by calculating the amount of absorbed verapamil. The capacity is 32.1 ± 5.2 nmol/$g_{(CMMBs)}$ ($n = 5$), representing the existence of calcium channel proteins immobilized onto the CMMBs. Calcium channel proteins are only one kind of membrane proteins laid on the RBC cellular membranes coated on the CMMBs, so the receptors used for the fishing assay is lower than that obtained by the other MB fishing assay with a known protein receptor [15–18], therefore, leading to the low fishing capacity. The low capacity may be solved by employing receptor-high-expression cells for the fishing assay in our future work.

Fishing from *Angelica dahurica*

Since cellular membrane proteins such as calcium channel proteins laid on the surface of CMMBs can interact with their ligand in the solution, the prepared CMMBs may be used for screening active compounds that could interact with the cellular membranes from natural product. *A. dahurica* was selected as the natural product. With the same as previous report [23], HPLC was also employed for analyzing the *A. dahurica* extract (Fig. 5a). The extract was incubated with the prepared CMMBs. The eluates were analyzed by the HPLC method previously reported [23] (Fig. 5b). By comparing the chromatograms of Fig. 5a, b, there were fewer peaks in Fig. 5b than that in Fig. 5a. The missing peaks may represent inactive components in *A. dahurica*.

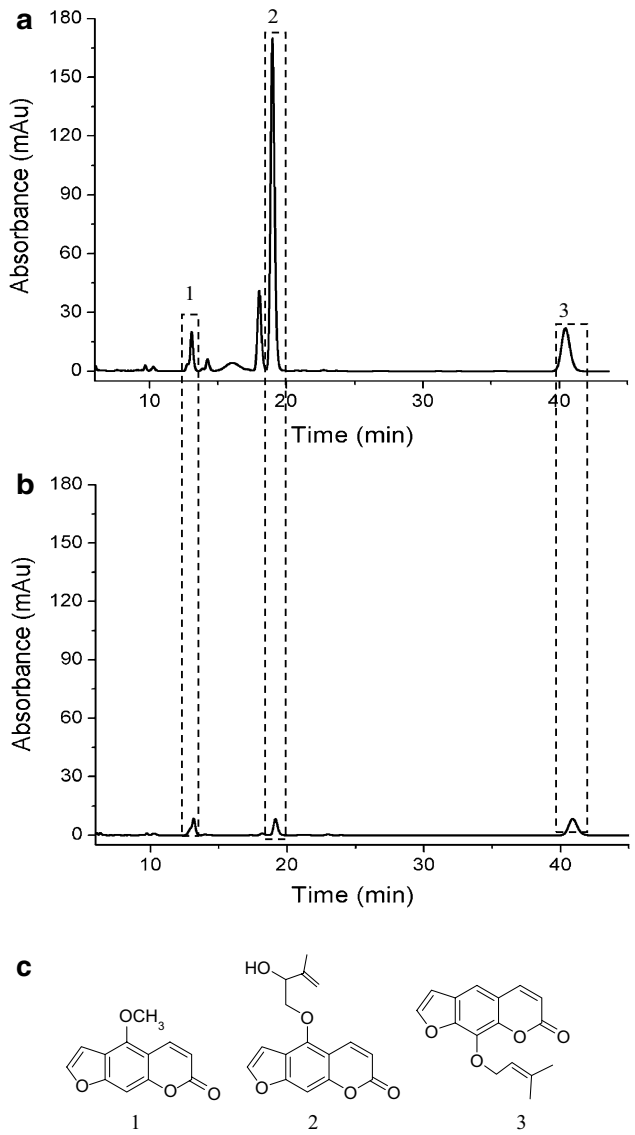

Fig. 5 Chromatograms of the *Angelica dahurica* extract (**a**) and the eluent from CMMBs after co-incubation with the extract (**b**). Chromatographic conditions are the same as that in Fig. 4. The structure of potential active compounds (compound 1 is bergapten, compound 2 is pabulenol, and compound 3 is imperation). The chromatographic conditions used for the analysis of eluates [*Column* Agilent Eclipse XDB C18 (4.6 × 250 mm, 5 μm); *Column temperature* 30 °C; *detective wavelength* 300 nm; *mobile phase* acetonitrile: 0.1% formic acid–water solution = 40:60; and *flow rate* 0.8 mL/min]

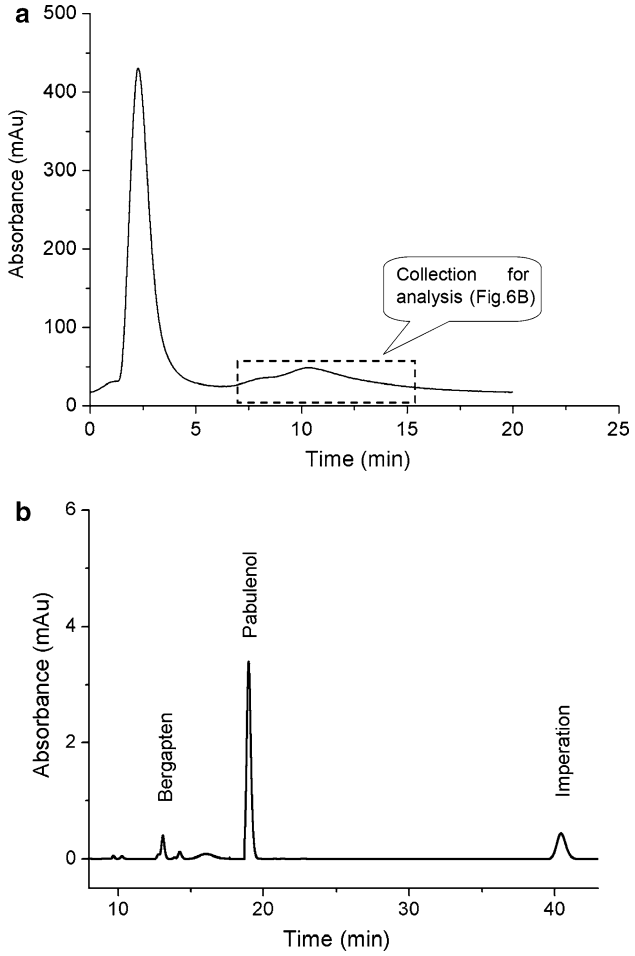

Fig. 6 Chromatogram of the extracts obtained by the CMC (**a**) and combined with offline HPLC system (**b**). **a** Injection volume. 1 μL of the extract (0.5 g crude drug/mL); flow rate, 0.1 mL/min; mobile phase, 10 mM PBS; and temperature 37 °C; **b** chromatographic conditions are the same as that in Fig. 5

The three active compounds shown in Fig. 5b were carefully characterized by MS or in comparison with the standard substance. Their chemical structures are demonstrated in Fig. 5c. Among which, imperation has been reported inhibiting voltage-dependent calcium channel and receptor-mediated Ca^{2+} influx and release [24]. Therefore, it can interact with calcium channel proteins located on the CMMBs and, therefore, be fished out. Peaks 1 and 2 in Fig. 5b were identified by high-resolution MS. Their molecular weights were 216.1921 and 286.2832, respectively. The MS results were the same as those of bergapten and pabulenol extracted from *A. dahurica* reported by Li et al. [25]. In addition, the retention time of peak 3 was also with the same as that of the standard imperation.

Since bergapten and pabulenol are analogues of imperation (Fig. 5c), so they may also interact with the calcium channel protein on the CMMBs. Detailed pharmacological activities of bergapten and pabulenol will be investigated in the future. The results shown in Fig. 5 suggest that the developed method is applicable for direct fishing of active compounds from a natural product.

It is noted from Fig. 5b that peak height of the fished compound is low compared to that in the extract (Fig. 5a). The peak height of the fished compound is depended on the corresponding membrane receptors' amount that can be used for ligand fishing. Therefore, the low peak height of

Table 1 Comparison of buffer as well as operation time between fishing with cellular membrane-coated magnetic beads (fishing with CMMB) and CMC

	Fishing with CMMB	CMC
Buffer (mL)[a]	<10	~400
Operation time		
Immobilization of cellular membranes[b] (h)	~12	~12
Fishing process[c] or chromatography process[d] (min)	~40	~130

Operation conditions were identical with Figs. 5 and 6, respectively

[a] Total consumption of buffer, including column washing, equilibration, and elution

[b] Including cell breaking, cell membrane coating, cell membrane fusing

[c] Including incubation (about 30 min) and elution (1–3 min)

[d] Including column equilibration (about 120 min), injection, and elution (at least 5 min)

Fig. 5b is attributed to the low CM capacity of the CMMBs. Therefore, developing new CMMBs embracing a high CMs' capacity should be a future direction of this method.

Comparison with CMC

To further assess possible advantages of using the developed CMMBs' fishing assay, CMC analysis of *A. dahurica* extract was also performed. The results are shown in Fig. 6a. Two chromatographic peaks were found. The peak marked in the figure was collected and analyzed by the HPLC for further identification. Figure 6b shows the chromatogram resulting from the HPLC analysis of the collected peak. The same potential active compounds (such as imperatorin, bergapten, and pabulenol) as that identified by the CMMBs' fishing assay were found. The result suggests that the CMMBs' fishing assay promises similar results to those obtained using the common CMC assay method in the screening of active compounds from natural products.

A further comparison between the CMMBs' fishing assay and CMC shows some apparent advantages in the use of the former. First, the use of buffered solution is also reduced in the fishing assay (Table 1). CMC requires an initial package of the column, followed by equilibration, and sample injection and elution. Hence, the CMC process consumed at least 400 mL of buffered solution for the analysis of *A. dahurica*. The analyzing process can also be simplified using the fishing method, as this consumed smaller amount of buffer (Table 1).

Second, the CMMBs' fishing method is time-saving. Although the operation time required for immobilizing CMs is similar, CMC always needs a relatively long time to equilibrate the column to obtain a flat baseline (about 2 h in our experiments). Therefore, CMC needs more operation time than the fishing method (Table 1).

Besides, the consumption of CMs in one CMC column could be reduced using CMMB fishing. A quarter of the CMs typically used for CMC are sufficient for one fishing assay. Though micro-CMC also needs relatively fewer CMs than common CMC [9, 19], yet the elution peaks from micro-CMC are difficult to collect for further analysis such as HPLC–MS.

Conclusions

In this work, CMMBs were for the first time used for a ligand fishing assay. With the RBC membrane-coated MBs, the potential active compounds such as imperation, bergapten, and pabulenol were fished out from *A. dahurica*. Compared with the CMC method, the developed fishing assay showed at least two advantages. First, less cell membranes and buffer are needed for the fishing assay, which fits the requirements of green analytical chemistry. Second, the CMMBs' fishing method is found to be more time-saving and easier in operation than the CMC technique. This is due to the fact that there is no need to spend a relatively long time to obtain a flat baseline (always needed for CMC) for the fishing assay. Furthermore, some operation procedures important for CMC success, such as column packing, sample, and buffer filtration and degassing, are unnecessary for the fishing method. Hence, the developed CMMBs' fishing assay can serve as an alternative method for screening active compounds from natural products.

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Compliance with Ethical Standards

Ethical approval All animal experiments have been approved by the Administrative Committee of Experimental Animal Care and Use of Tianjin Medical University and, furthermore, conformed to the guidelines set by the National Institute of Health on the ethical use of animals.

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