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Preparation of Molecularly Imprinted Composite Membranes for Inducing Bergenin Crystallization in Supercritical CO_2 and Adsorption Properties

Wencheng Zhang,* Qing Zhang,† Ruixia Wang, Yanfang Cui, Xingyuan Zhang,† and Lile Hong

Engineering Research Center of Bio-process, Ministry of Education, Hefei University of Technology, Hefei 230009, P.R. China
**E-mail: zwc1012@163.com*
†CAS Key Laboratory of Soft Matter Chemistry, Department of Polymer Science and Engineering, University of Science and Technology of China, Hefei 230026, P.R. China
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The concept of molecular imprinting was first reported by Wullf and his cooperators, which is a method for introducing molecular recognition properties to the functional polymers synthesized in the presence of template molecules.[1] Molecular imprinting is becoming an attractive selective recognition method and has been evaluated for a wide variety of applications including separation, catalysis, sensors, and antibodies.[2-6] Generally, the process of molecular imprinting is composed of three steps: (a) covalent conjugate or non-covalent adduct between a functional monomer and a template molecule, which is the preorganization step; (b) polymerization of this monomer-template conjugate (or adduct) and (c) removal of the template from the polymer.[7] In the above procedures, the molecular memory is strongly dependent on the formation and status of the template-monomer preorganization conjugate (or adduct). Therefore, to study these conjugates/adducts in detail is crucially important for understanding the imprinting mechanism and designing efficient molecular imprinting systems.[8-10]

On the other hand, one of the most important and facile ways to realize the molecular imprinting is using the molecularly imprinted membranes (MIMs), which were first introduced by Piletsky *et al*..[11] In MIMs systems, the combination of the imprinting technique can provide membranes with specific selectivity for the separation of targeted organic compounds and thus make the MIMs possess the advantages of both molecular imprinting and membrane technology.[12-14] According to the preparation methods, MIMs can be generally divided into three categories, namedly molecularly imprinted filling membranes (MIFMs), molecularly imprinted monolithic membranes (MIMMs), and molecularly imprinted composite membranes (MICMs),[15] among which MICMs attract more attention because of their high flux of the resulted composite membranes and well flexibility of applications.[6]

As one of chemical engineering processes, supercritical fluid extraction (SCFE), nowadays, has widely been used to separate and purify natural herbal products.[16,17] Supercritical fluids, especially $ScCO_2$, are used as processing media in more complex applications such as crystal growth,[18] anti-solvent precipitation,[19] reaction,[20] dyeing,[21] and so on. Processes that involve supercritical fluid are of great interest for the crystallization of compounds difficult to be separated. Because of their high diffusivity, high solvent power and low viscosity, supercritical fluids are very interesting solvents, allowing improved mass transfer.[22]

In the present work, Bergenin, isocoumarin compound,[23] was chosen as the target molecule to synthesize MICMs by UV initiated photo-copolymerization using polysulfone (PSF) ultrafiltration membranes as porous supports. The thin imprinted layers deposited on the surface of the support membranes were formed by copolymerization of acrylamide (AM) as functional monomer, ethylene glycol dimethacrylate (EGDMA) as cross-linker and 2-2'-azobisisobutyronitrile (AIBN) as initiator in the presence of Bergenin as template molecule in *N,N*-dimethylformamide (DMF) solution. The optimum conditions for preparation of Bergenin-molecularly imprinted composite membranes (Bergenin-MICMs) were obtained as follows, the molar ratio of Bergenin, AM and EGDMA 1:4:12, the elution time 6 h, the amount of photoinitiator 1.5% and the concentration of Bergenin 1 mmol/L, respectively. Scanning electron microscope (SEM) was utilized to visualize surface of membranes to gain better understanding in the analysis of imprinted layers deposited on PSF support membranes. The inducing crystallization capacity of the resultant Bergenin-MICMs in supercritical CO_2 ($ScCO_2$) was investigated. The crystallization rate of Bergenin reached 44.7%, and the purity of Bergenin was 98.1%. The results of this study indicated that the synthesized molecularly imprinted composite membranes have good specific adsorption properties and capacity to induce crystallization of Bergenin in $ScCO_2$.

Five kinds of solution in which the molar ratios of Bergenin to AM were 1:1, 1:2, 1:4, 1:6 and 1:8 were prepared by adding different amounts of AM into 8.4 mmol/L Bergenin DMF solution. It was concluded that with the increase of concentration of AM, the amounts of Bergenin bound to the AM increased gradually, while the molar ratio reached 1:4, the value of Q (Q, mg/cm^2, calculated by subtracting the amounts of free Bergenin from the amounts

of Bergenin initially added) stabilized. When the molar ratio was more than 1:4, excessive functional monomer could produce residues of non-assembly functional monomer, which made non-selective binding sites increase. On the other hand, when too many functional monomers were added, excessive of them would cross-linked with each other which resulted in the decreasing of selective binding sites. Therefore, the optimal molar ratio of Bergenin to AM was 1:4.

Four imprinted composite membranes were prepared under the conditions that the molar ratios of Bergenin, AM and EGDMA were 1:4:8, 1:4:12, 1:4:16, 1:4:20, respectively. The prepared Bergenin-MICMs were put into the 25 mL DMF solution which contained 1 mmoL Bergenin, and soaked for 1 h in order to measure the binding amounts. It was found that with the increase of EGDMA, the binding amounts first increased, then decreased. And when the molar ratio of Bergenin, AM and EGDMA was up to 1:4:12, the binding amount was optimal. It was implied that excessive EGDMA might form gel and when the concentration of EGDMA was too low, the polymer could not form homogeneous and stable cavities and identify target molecule.

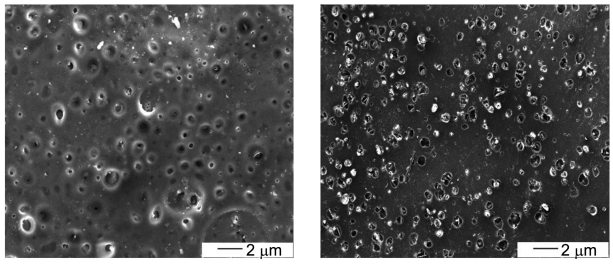
Bergenin-MICMs were eluted with 300 mL methanol to remove the template molecule Bergenin. It was found that the absorbance increased gradually with the increase of elution time, and after six hours the absorbance basically stabilized. Then the Bergenin-MICMs were placed in pure methanol, eluted for 2 h, the absorbance value was zero. So elution time was selected as 6 h.

Photoinitiators of 0.5%, 1.0%, 1.5% and 2.0% were added into the casting solution to prepare the MICMs. It was concluded that the binding amounts of MICMs to Bergenin increased gradually as the increase of the concentration of photoinitiator. When the concentration of photoinitiator was up to 1.5%, the binding amount was largest.

In order to investigate the effect of the concentration of Bergenin on preparation of Bergenin-MICMs, different concentrations of Bergenin were added to prepare Bergenin-MICMs according to the method mentioned above. The results showed that within the range of 1-10 mmol/L, the binding capacity of Bergenin to the resultant membranes was relatively close to each other. Taking conservation of raw materials into account, 1 mmol/L was chosen to prepare Bergenin-MICMs.

For morphology observation of the resultant membranes, SEM was used. In Figure 1, (a) showed that few cupped pores were observed on the surface of MICMs before template molecule Bergenin being extracted; (b) The surface of MICMs was significantly changed after the extraction of Bergenin, the "molecularly imprinted" cavities formed in these MICMs are distinct round shaped. Evidently, the presence of the template molecule, which does not take part in the formation of the network, might work as an obstacle for its formation.[24]

Crystallization as a process that solute precipitated from solution could be divided into two stages such as formation of crystal nucleus and growing of crystals. The type of



(a) before extraction (b) after extraction

Figure 1. SEM microphotographs of MICMs.

crystal was determined by the crystal nucleus. The formation of crystal nucleus had three forms, including the initial homogeneous nucleation, the initial non-homogeneous nucleation and secondary nucleation. At present, the crystallization operation had been widely used in many chemical products, such as dyestuffs, paints and pharmaceuticals. However, the traditional crystallization process with residual solvent would not only affect the quality of products, but also endanger human health. Many researchers had studied a lot about supercritical CO_2 crystallization because of its safety and free solvent residue. But the crystallization process of supercritical CO_2 had bad specificity, and could not obtain a kind of high purity compound by a single crystallization. Molecularly imprinted polymers had very good specificity and could withstand high pressure, so the crystallization process of supercritical CO_2 was combined with molecularly imprinted technique to improve the specificity of its crystallization in this paper. We put the Bergenin-MICMs and Bergenin-NMICMs in the extractor of supercritical fluid extraction system and added 10 mL saturated solution of Bergenin-methanol at 40 °C. The extraction pressure and temperature were controlled at 10 MPa, 40 °C, and extracted circularly for 90 min. The crystals were collected from the plates after the end of experiments. The purity of crystals was measured by HPLC-UV (Fig. 2) and the crystallization rate was calculated by the percentage of crystals to raw materials. The results were shown in Table 1. It can be seen that the Bergenin were formed to crystals by adding MICMs and NMICMs plates in supercritical CO_2, and the purity was improved. But the purity and crystallization rate of Bergenin induced crystallization by MICMs were higher than those of NMICMs in supercritical CO_2. It

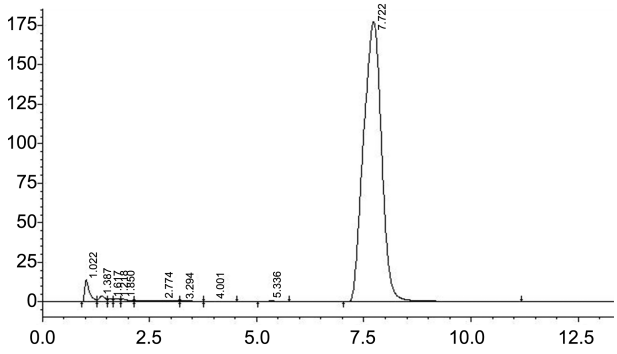


Figure 2. HPLC-UV chromatograms of Bergenin (purity of 98.1%).

Table 1. The purity and crystallization rate of Bergenin inducing crystallization in supercritical CO_2

Crystal template	Purity (%)	Crystallization rate (%)
Bergenin-MICMs	98.1	44.7
Bergenin-NMICMs	84.3	38.9

could be understood that the binding sites provided by the holes complementary with the structure of Bergenin on the MICMs surface made the formation of Bergenin crystal nucleus rapidly in supercritical CO_2 crystallization process and then the Bergenin crystal grew gradually. The orientation and specificity of crystallization generated, so the purity and crystallization rate of Bergenin were improved greatly.

Experimental Section

Materials and Methods. Bergenin raw material (with the purity of Bergenin 70.0%) was provided by Tuofeng Co. of Anhui Province. The Bergenin standard (purity ≥ 99%) was supplied by National Institute for the Control of Pharmaceutical and Biological (Beijing, China). Acrylamide (AM) and ethylene glycol dimethacrylate (EGDMA) were purchased from Shanghai Co-founder Chemical Ltd. (Shanghai, China). 2-2'-Azobisisobutyronitrile (AIBN) was supplied by Beijing Chemical Reagent Company (Beijing, China). *N,N*-Dimethylformamide (DMF), methanol and acetic acid were bought from Sinopharm Chemical Reagent Ltd. (Shanghai, China). Polysulfone (PSF) ultrafiltration membranes used as porous supports for the deposition of polymer layer were purchased from Dalian Polysulfone Plastics Ltd. (Dalian, China). EGDMA and AM were distilled under reduced operation pressure to remove inhibitor and AIBN was recrystallized with methanol before use. Other chemicals (analytical grade) were utilized without further purification. Our self-made supercritical fluid crystallization system with 2.5 L crystallization reactor and UV-1600 spectrophotometer (Tsingtao Unicom-Optics Instruments Co., Ltd., China) were used in this study. For morphology observation of the resultant membranes, a scanning electron microscope (SEM, Hitachi X-650, Japan) was used. The purity of Bergenin was determined by HPLC-UV (Waters 2487, USA).

Preparation of Membranes. Using Bergenin as template, AM as functional monomer and EGDMA as cross-linker, the MICMs were prepared by UV initiated copolymerization with PSF ultrafiltration membrane as porous support between two glass plates. A typical preparation process was carried out as follows (Fig. 3): To produce the imprinted polymer layer of molecularly imprinted composite membrane, 0.1 mmol template Bergenin was dissolved with 0.4 mmol monomer AM under ultrasonic condition in 15 mL DMF in a 50 mL conical flask, and then, 0.1 mmol free radical initiator AIBN and 1.6 mmol cross-linker EGDMA were added into the solution. After deoxygenating with nitrogen for 10 min, the supporting membranes were coated by soaking in the solution mentioned above for 30 min. Then, the membranes, saturated with the mixed solution on the

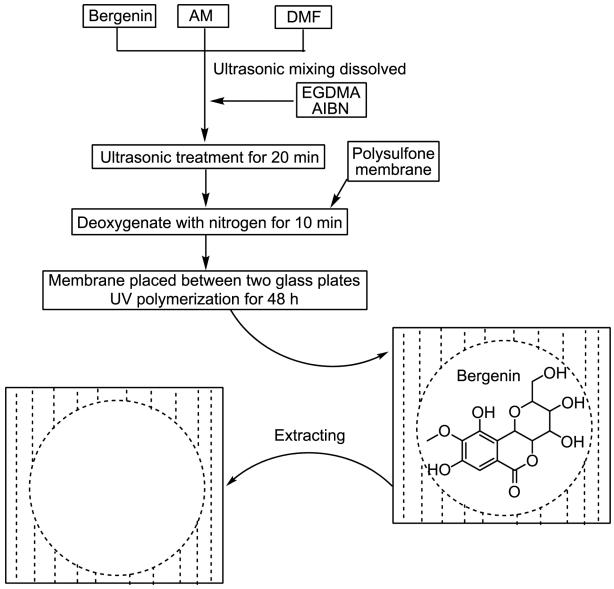


Figure 3. Schematic illustration of Bergenin imprint process.

binding sites of the surface, were taken out from the flask and deposited between two glass plates immediately. The glass plates were exposed to a UV lamp at a relative radiation intensity of 20 W/m^2 and wavelength of 365 nm for 48 h at room temperature. After polymerization completed, the composite membranes obtained were eluted with methanol/acetic acid (9:1, v/v) and distilled water to remove the template and any nonpolymerized compounds until there was no Bergenin that could be detected by HPLC-UV in the eluent. When template was thoroughly eluted, the composite membranes were dried to constant weight in a vacuum drying oven at 50 °C, preserved for further utilization. The non-imprinted composite membranes were prepared following the procedures but in the absence of Bergenin.

Inducing Crystallization in Supercritical CO_2. MICMs were uniformly mixed with the adhesive (CMC), coated on the glass plate (15 cm × 5 cm), and dried at 60 °C for 2 h in the oven. The non-molecularly imprinted membrane plates were prepared by the same method except that the Non-molecularly imprinted composite membranes (NMICMs) were used. Then the molecularly imprinted membrane plates were put in the crystallization reactor of supercritical fluid crystallization system and 10 mL Bergenin-methanol saturated solution was added. The crystallization pressure, crystallization temperature and crystallization time were controlled at 10 MPa, 40 °C and 90 min, respectively. When the crystallization process was completed, Bergenin crystals were collected from the molecularly imprinted thin-layer plates, and the purity was measured with HPLC-UV.

Analysis of Bergenin. The chromatographic system consisted of a model 515 HPLC pump and a Waters 2487 dual λ absorbance detector. All separations were achieved on Resteck C18 reversed-phase column (250 mm × 4.6 mm, 5 μm). The mobile phase was methanol and distilled water (80:20, v/v). The column temperature was kept at 25 °C with a flow-rate of 1 mL/min; and the UV detector wavelength

was set at 275 nm. The injection volume was 10 μL. The Empower software was used to acquire and process the chromatographic data. A series of concentrations of standard solution were prepared with Bergenin standard and the standard curve was obtained by HPLC. The result presented a very good linear relationship between the sample volume of Bergenin and the peak area, with the correlation coeffcients (r^2) of 0.9997 in the range of 1-18 μg.

The purity of Bergenin was calculated as follows:

$$\text{Purity} = W_1/W_2 \times 100\% \quad (1)$$

where W_1 (g) was the weight of Bergenin detected by HPLC, W_2 (g) was the weight of Bergenin after crystallization.

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