Analytical Methods

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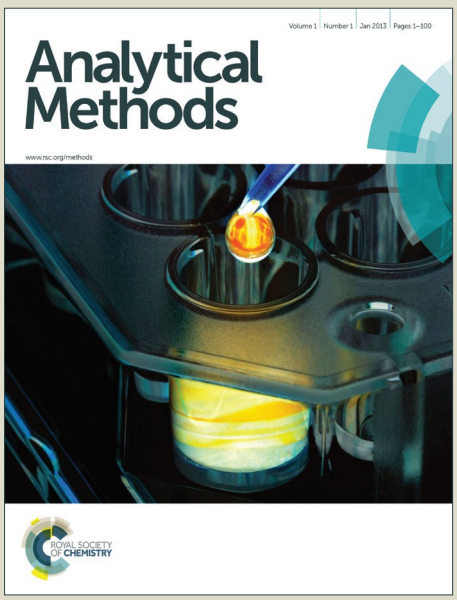

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ARTICLE

Preparation and evaluation of molecularly imprinted composite membranes for inducing crystallization of oleanolic acid in supercritical CO_2

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Wencheng Zhang, [a] Qing Zhang, [b] Xingyuan Zhang,[b,*] Zeyu Wu, [a,*] Bing Li, [a] Xiuli Dong [a] and Bingsong Wang [a]

In this study, novel molecularly imprinted composite membranes (MICMs) for the inducing crystallization of oleanolic acid (OA) in supercritical CO_2 ($ScCO_2$) have been described. The MICMs were synthesized by the UV initiated photo-copolymerization, with OA as a template molecule, copolymerization of methacrylic acid (MAA) as a functional monomer, ethylene glycol dimethacrylate (EGDMA) as a cross-linker and polysulfone (PSF) ultrafiltration membranes as porous supports. The preparation conditions of OA-MICMs were optimized as follows, the molar ratio of OA, MAA and EGDMA 1:4:12, the amount of photoinitiator 1.5%, the concentration of OA 1 mmol/L and the elution time 6 h, respectively. Scanning electron microscopy (SEM) was used for characterization of MICMs. The MICMs were used for inducing crystallization of OA in $ScCO_2$. The purity and the crystallization rate of OA were 98.3% and 45.3%, respectively. Our work presents a new method to induce crystallization of OA in $ScCO_2$ by using MICMs, with specific adsorption properties and capacities.

Introduction

Oleanolic acid (OA), an important kind of pentacyclic triterpenoid, is widely existed in medical herbs and other plants in the form of free body and glycosides.[1] Recently, more and more attentions are paid to OA, due to its pharmacological activities, such as anti-inflammation,[2-4] antivirus,[5] antitumor,[6-9] hepatoprotective,[9-11] hypolipidemic and hypoglycemic effects.[12] The synthetic route of OA has not yet been reported owing to its complicated chemical structure, so the main source of OA is still extracted from plants. Several conventional methods,[13-18] including solvent extraction, ultrasonic-assisted extraction, microwave-assisted extraction, ultra high pressure extraction, macroporous resin and silica gel column have been developed for the extraction and purification of OA. However, these methods had some disadvantages, such as complex operation steps, abundant solvent consumption and serious environmental problem.[19,20] In addition, in many kinds of natural products, OA is always accompanied by ursolic acid (structure shown in Fig.1), which has the similar structure to OA. Therefore, effective separation and purification technology for OA with high purity is extremely important and urgently needed.

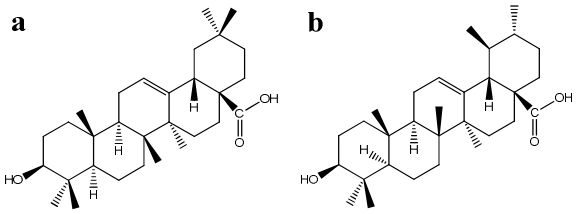

Fig.1 Chemical structures of OA (a) and ursolic acid (b).

Two new techniques, molecular imprinted membranes (MIMs) and supercritical fluid crystallization (SCFC) are particularly suitable for separation and purification of natural products. As firstly reported by Wullf and Sarhan in 1972,[21] molecular imprinting technology (MIT) was a preparation process of polymers with high selectivity, which could be divided into three steps, i.e., covalent conjugate or non-covalent adduct between the template molecule and functional monomers, copolymerization of template–monomer conjugate (or adduct), and removal of template from the polymer.[22] Because of various characteristics, including easy preparation, good specificity, low cost, compatibility with organic solvents, tolerance to a wide range of temperature, pressure and pH value, MIT has been widely applied in separation, catalysis, sensors, antibodies and other fields.[23-29] With the development of MIT, MIMs were firstly introduced by Piletsky in 1990,[30] and possessed the advantages of both MIT and membrane technology, providing membranes with specific selectivity for the separation of targeted compounds.[31-33] Compared with conventional

a Engineering Research Center of Bio-process, Ministry of Education, Hefei University of Technology, Hefei 230009, PR China

b 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
[*] *E-mail: zxym@ustc.edu.cn, wuzeyu@hfut.edu.cn; Tel:+86 551 62901826; Fax: +86 551 62901862.*

molecularly imprinted polymer particles (MIPPs), molecularly imprinted composite membranes (MICMs)[34] have shown simple preparation process without grinding, small diffusion resistance, high flux and flexible applications.[27,35,36]

On the other hand, crystallization is very effective for the purification of compounds that were difficult to be purified. Traditional crystallization process with residual solvent would not only affect the quality of products, but also endanger human health.[37,38] As an ideal solvent, CO_2 is generally used in SCFC instead of organic solvent because of high diffusivity, non-toxicity, low viscosity and easy removal from the extracts.[39,40] In addition, low temperature during the SCFC process could reduce the loss of thermal sensitive components.[41-43] However, SCFC has bad specificity, and could not obtain a kind of high purity compound by a single crystallization. Therefore, SCFC combined with MICMs is expected to improve the purification efficiency of compounds.

In this study, based on the advantages of MIT and SCFC, novel MICMs for selective binding and separation of OA from complicated systems were first prepared, followed by the surface functionalized imprinting with UV initiated photo-copolymerization, using OA as a template molecule, copolymerization of methacrylic acid (MAA) as a functional monomer, ethylene glycol dimethacrylate (EGDMA) as a cross-linker and polysulfone (PSF) ultrafiltration membranes as porous supports. After characterization by scanning electron microscope (SEM), the OA-MICMs were used for inducing crystallization of OA in supercritical CO_2 ($ScCO_2$) to obtain highly pure OA.

Experimental

Materials and chemicals

OA (purity ≥ 99%) was supplied by Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). PSF ultrafiltration membranes used as porous supports for the deposition of polymer layer were purchased from Dalian Polysulfone Plastics Ltd (Dalian, China). MAA and EGDMA were bought from Shanghai Co-founder Chemical Ltd. (Shanghai, China) and distilled under reduced operating pressure to remove inhibitor. 2-2'-azobisisobutyronitrile (AIBN) was obtained from Beijing Chemical Reagent Company (Beijing, China) and recrystallized with methanol. Analytical grade chloroform, methanol and acetic acid were purchased from Sinopharm Chemical Reagent Ltd. (Shanghai, China) and utilized without further purification. Nitrogen and carbon dioxide gas (purity ≥ 99.99%) were supplied by Jinwang Gas Limited Company (Hefei, China).

Preparation of OA-MICMs and NICMs

To produce the imprinted polymer layer of MICMs, 0.1 mmol OA (template) and 0.4 mmol MAA (monomer) were dissolved with 25 mL chloroform (or methanol) in a 50 mL conical flask under ultrasonic condition and standed for 6 h. Then, 0.1 mmol AIBN (free radical initiator) and 1.6 mmol EGDMA (cross-linker) were added into the solution and mixed by ultrasonic treatment for 20 min. After deoxygenation with nitrogen for 10 min, the supporting membranes were coated by soaking in the above solution for 30 min. Subsequently, the membranes, saturated with the mixed solution on the 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 MICMs obtained were eluted with methanol/acetic acid (9:1, v/v) and distilled water to remove the template and any nonpolymerized compounds until no OA could be detected by HPLC-UV in the eluent. When the template was thoroughly eluted, the composite membranes were dried to constant weight in a vacuum drying oven at 50°C, and preserved for further utilization. The detailed preparation process was shown in Fig. 2.

The non-imprinted composite membranes (NICMs) were prepared following the same procedures but in the absence of OA.

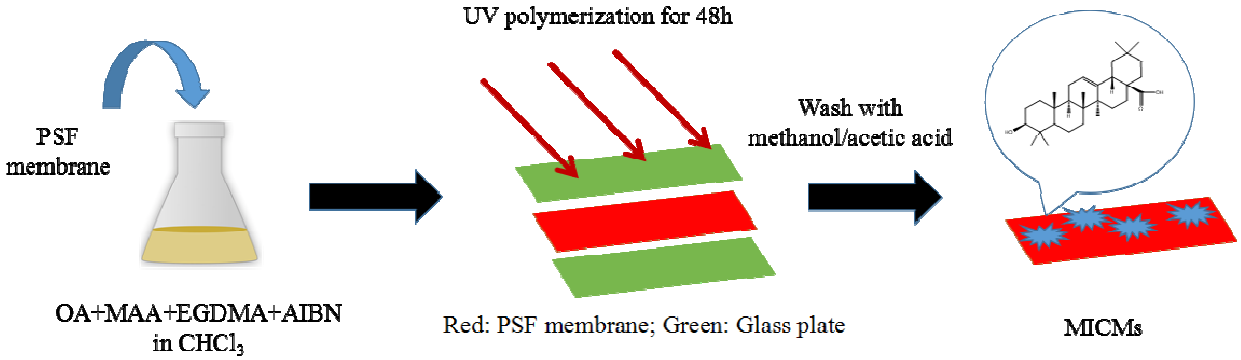

Fig. 2 Process for preparation of MICMs.

Optimization of OA-MICMs preparation

For the optimization of preparation process of OA-MICMs, the effect of different molar ratios of OA to MAA (1:1, 1:2, 1:4, 1:6 and 1:8), molar ratios of OA, MAA and EGDMA (1:4:8, 1:4:12, 1:4:16 and 1:4:20), elution time (1h, 2h, 3h, 4h, 5h, 6h and 7h), concentration of photoinitiator (0.5%, 1.0%, 1.5% and 2.0%) and concentration of OA (0.5, 1, 5, 10 and 15 mmol/L) were investigated, respectively.

Characterization of OA-MICMs

The morphology of PSF ultrafiltration membranes, OA-MICMs prepared in methanol and OA-MICMs prepared in chloroform was observed by SEM (Hitachi X-650, Japan).

Inducing crystallization in $ScCO_2$

After MICMs or NICMs uniformly mixed with carboxymethyl cellulose (CMC, adhesive), the mixture was coated on the glass plate (15 cm × 5 cm), and dried in the oven for 2 h at 60 °C. Then, the plates were put into a 2.5 L crystallization reactor of our self-made supercritical fluid crystallization system (shown in Fig. 3) with addition of 10 mL OA-methanol saturated solution. Subsequently, the crystallization process was carried out with the pressure, temperature and time of 13 MPa, 42 □and 2 h, respectively. After the crystallization process was completed, OA crystals were collected from the molecularly imprinted thin-layer plates, and the purity was measured with HPLC-UV.

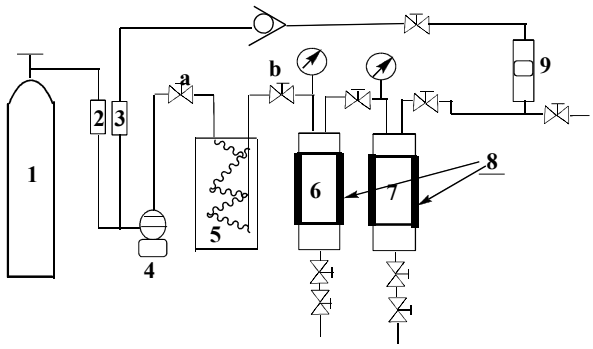

Fig. 3 Flow chart of OA crystallization in $ScCO_2$. (1. Gas vase; 2, 3. Filter; 4. Compressor; 5. Hot exchanger; 6. Crystallization kettle; 7. Separation kettle; 8. Water bath; 9. Flowmeter; a, b. Booter).

High performance liquid chromatography (HPLC) analysis of OA

The HPLC analysis was carried out with the chromatographic system (Waters, USA) equipped with a model 515 HPLC pump and a 2487 dual λ absorbance detector. All the separations were achieved on a Resteck C_{18} reversed-phase column (250 mm × 4.6 mm, 5 μm) with a flow-rate of 1 mL/min at 30 °C. The mobile phase consisted of methanol and distilled water (85:15, v/v). The UV detector wavelength was set at 210 nm and injection volume was 10 μL.

A series of concentrations of standard solution were prepared with OA standard and the standard curve was obtained by HPLC. The result presented a very good linear relationship between peak area and sample volume of OA, with the correlation coeffcient (r^2) of 0.9965 in the range of 0.04-0.8 mg/mL. The purity of OA was calculated as follows:

$$\text{Purity} = W_1/W_2 \times 100\%$$

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

Results and discussion

Effect of molar ratio of OA to MAA on adsorption capacity of OA-MICMs

Solutions with different molar ratios of OA to MAA (1:1, 1:2, 1:4, 1:6 and 1:8) were prepared by adding different amounts of MAA into 8.4 mmol/L OA chloroform solution. According to the preparation process mentioned above, OA-MICMs adsorbed OA from chloroform solution. The amounts of OA binding to OA-MICMs (Q, mg/cm^2) were calculated by subtracting the amounts of free OA from the amounts of OA initially added.

The effect of molar ratio of OA to MAA on adsorption capacity of OA-MICMs was shown in Fig.4. The adsorption capacity rose gradually with the increase of concentration of MAA and stabilized at the molar ratio of 1:4. When the molar ratio was less than 1:4, excessive functional monomer MAA could produce residues of non-assembly functional monomer, leading to the increase of non-selective binding sites. In addition, when too many functional monomers were added, excessive of them would be cross-linked with each other, which resulted in the decrease of selective binding sites.[44] Therefore, 1:4 was selected as the optimal molar ratio of OA to MAA.

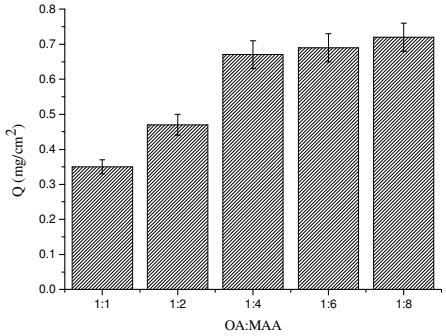

Fig. 4 Effect of molar ratio of OA to MAA on adsorption capacity of OA-MICMs prepared in chloroform (Note: 1:4 was selected as the optimal molar ratio of OA to MAA).

Effect of amount of EGDMA on adsorption capacity of OA-MICMs

The appropriate molar ratios of template, functional monomer and cross-linker are so important to enhance the number of MICMs recognition sites. The ratio of template, functional monomer and cross-linker was optimized as follows. Four imprinted composite membranes were prepared under the conditions that the molar ratios of OA, MAA and EGDMA were 1:4:8, 1:4:12, 1:4:16, 1:4:20, respectively. After the prepared OA-MICMs put into 25 mL methanol solution containing 1 mmoL OA, and soaked for 1 h, the binding amounts were measured. As shown in Fig. 5, with the increase of EGDMA, the binding amounts first increased and

then decreased. The highest adsorption capacity was observed when the molar ratio of OA, MAA and EGDMA was up to 1:4:12, implying that excessive EGDMA might form gel and when the concentration of EGDMA was too low, the polymers could not form homogeneous and stable cavities and identify target molecule.

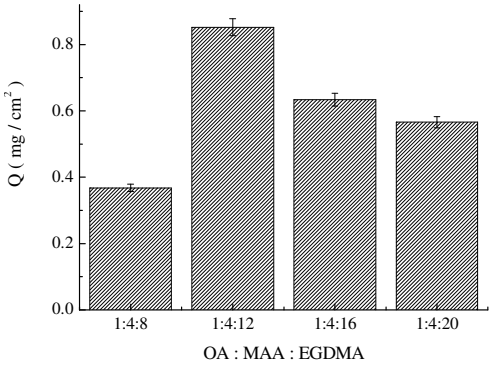

Fig. 5 Effect of the amount of EGDMA on adsorption capacity of OA-MICMs prepared in chloroform (Note: 1:4:12 was selected as the optimal molar ratio of OA, MAA and EGDMA).

Effect of photoinitiator concentration on adsorption capacity of OA-MICMs

Four different concentrations of photoinitiator (0.5%, 1.0%, 1.5% and 2.0%) were added into the casting solution to prepare the MICMs. The results (Fig.6) showed that the binding amount of MICMs to OA increased gradually as AIBN concentration rose from 0.5% to 1.5%, but decreased from 1.5% to 2%. In brief, the effect of AIBN concentration on adsorption capacity of OA-MICMs was not obvious. As a result, 1.5% was used as the concentration of photoinitiator.

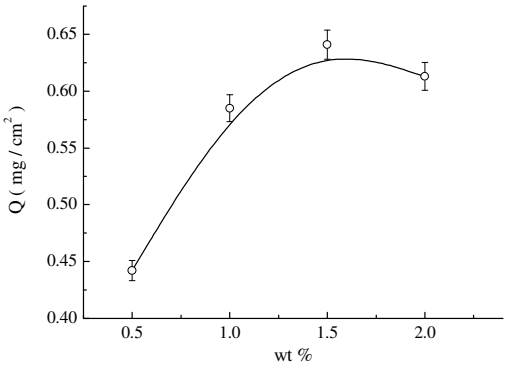

Fig. 6 Effect of AIBN concentration on adsorption capacity of OA-MICMs prepared in chloroform.

Effect of OA concentration on adsorption capacity of OA-MICMs

In order to investigate the effect of OA concentration on preparation of OA-MICMs, different concentrations of OA were added to prepare OA-MICMs according to the method mentioned above. In Fig.7, it could be found that the binding amount of MICMs to OA significantly rose as OA concentration from 0.5 mmol/L to 1 mmol/L. When OA concentration was within the range of 1-15 mmol/L, the binding capacity of OA to resultant membranes was relatively close to each other. Taking conservation of raw materials into account, 1 mmol/L of OA was chosen to prepare OA-MICMs.

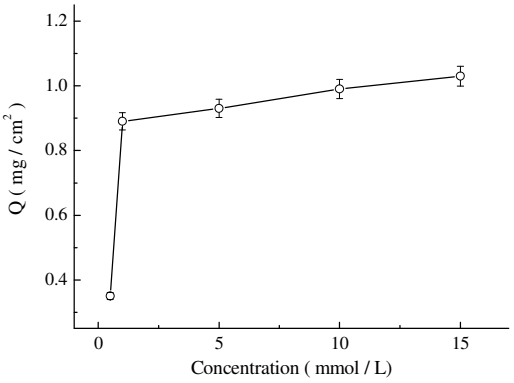

Fig. 7 Effect of OA concentration on adsorption capacity of OA-MICMs prepared in chloroform.

Effect of elution time for OA-MICMs

After OA-MICMs successfully being prepared, OA (template molecule) should be removed from the polymers, to form effective imprinted sites. During the elution process, the concentration of OA in eluents was determined by UV-1600 spectrophotometer (Tsingtao Unicom-Optics Instruments Co., Ltd., China).

OA-MICMs were eluted with 300 mL methanol to remove the template molecules OA. Fig. 8 showed that the absorbance increased gradually with the increase of elution time, and after 6 h the absorbance was basically stabilized. Then the OA-MICMs were placed in pure methanol, eluted for 2 h, the absorbance value was zero. Therefore, elution time was identified as 6 h.

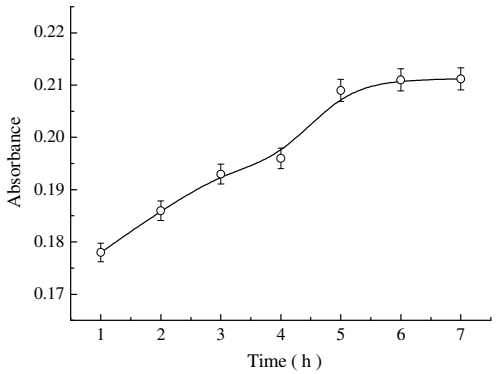

Fig. 8 Effect of elution time on absorbance of OA-MICMs prepared in chloroform.

Morphology observation of membranes

As we know, the interaction between the template molecule and the functional monomer, and then the adsorption properties of MICMs, could be affected by the polarity of porogenic solvents. Therefore, SEM was employed to capture the detailed morphology of PSF membrane, OA-MICMs prepared in methanol and OA-MICMs prepared in

chloroform, respectively. As shown in Fig. 9 (a), the surface of basement membrane was dense and smooth. And after graft on basement membrane, the surface of OA-MICMs was relatively dense. The surface of OA-MICMs prepared using methanol as solvent (Fig. 9 (b)) was tooth-like dense, while that of OA-MICMs prepared using chloroform as solvent (Fig. 9 (c)) was in chains. It was indicated that the surface of blank PSF support membrane was covered by a thin imprinted layer after polymerization procedure. Moreover, methanol could also form hydrogen bond with functional monomer to make the pores irregular and specific selectivity reduced. The above results are consistent with that reported by the literature.[28]

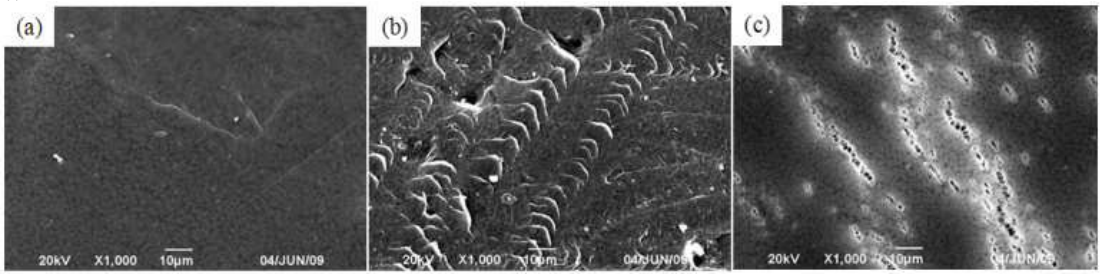

Fig. 9 Scanning electron micrographs of PSF membrane (a), OA-MICMs/methanol (b) and OA-MICMs/chloroform (c).

OA-MICMs inducing crystallization in $ScCO_2$

After crystals collected from the plates at the end of experiments, the purity of crystals was measured by HPLC-UV (Fig. 10) and the crystallization rate was calculated by the percentage of crystals to raw materials.

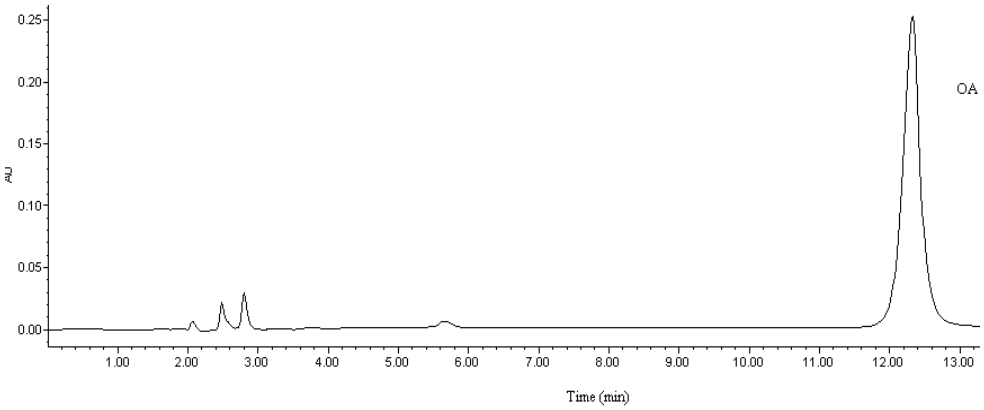

Fig. 10 HPLC-UV chromatogram of OA (purity of 95.3%).

According to the results shown in Table 1, it can be seen that the OA was formed to crystals by adding NICMs and MICMs plates in $ScCO_2$. Compared with NICMs, the purity and crystallization rate of OA induced crystallization by MICMs were higher. It could be understood that the binding sites provided by the holes complementary with the structure of OA on the MICMs surface made the formation of OA crystal nucleus rapidly in $ScCO_2$ crystallization process and then the OA crystal grew gradually. The orientation and specificity of crystallization generated, so the purity and crystallization rate of OA were improved greatly.

Compared with the crystallization behaviors reported in our previous study, in which the purity and crystallization rate of OA were 95.7% and 93.1% by using molecularly imprinted polymers (MIPs), the crystallization rate was decreased, but the purity of OA was improved. And MICMs have shown simple preparation process, high flux and flexible applications. Moreover, how to improve the crystallization rate of MICMs will be an interesting research direction in the future.

Table 1 The Purity and crystallization rate of OA induced crystallization by NICMs and MICMs in $ScCO_2$

Membrane	Purity	Crystallization rate
OA-NICMs	90.6%	39.4%
OA-MICMs	98.3%	45.3%

Conclusions

In summary, MICMs were successfully prepared for the inducing crystallization of OA in $SCCO_2$. The optimized preparation conditions of MICMs were described as follows, the molar ratio of OA, MAA and EGDMA 1:4:12, the amount of photoinitiator 1.5%, the concentration of OA 1 mmol/L and the elution time 6 h. The characterization results of SEM suggested that, compared with methanol, chloroform should be selected as solvent for the preparation of MICMs. The prepared MICMs have very good performance for the inducing crystallization of OA in $SCCO_2$, with the crystallization rate and purity of 45.3% and 98.3%, respectively. All these results demonstrate that this method is

feasible and rapid for the preparation of high pure active ingredients from natural products.

Acknowledgment

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Graphical Abstract

In this study, molecularly imprinted composite membranes (MICMs) were successfully prepared and used for inducing crystallization of oleanolic acid (OA) in supercritical CO_2.