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Metabolic Fingerprints by Nano-baskets of 1,2-Alternate Calixarene and Emulsion Liquid Membranes

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A novel approach for metabolite extraction and fingerprinting was introduced based upon the nano-baskets and emulsion liquid membrane-nuclear magnetic resonance (ELM-NMR) technique. The objective of this method is optimizing the fingerprints, minimizing the metabolic variation from analysis, increasing the likelihood differences, and obtaining the maximum extraction yield. Low molecular weight metabolites in rat serum were recovered by ELMs using 12 nano-baskets of calixarene, as both emulsifier and carrier. The yields of ELMs were optimized by the method of one-at-a-time. According to NMR data, the maximum metabolic variation was achieved using scaffold **4** (4 wt %), *n*-decane membrane, stirring rate of 300 rpm, treat and phase ratios of 0.3 and 0.8, respectively. The results revealed that some calixarenes tend to extract non-specific macromolecules; and repeatability of fingerprints for **7**-mediated ELM was maximum and for **3**-mediated ELM was minimum. The yield of extractions was obtained to be higher for *n*-decane and lower for carbon tetrachloride. Among different membranes, the fingerprints by chlorinated liquid membranes were more repeatable than using toluene or *n*-decane.

Key Words : Nano-baskets, Metabolomics, Emulsion liquid membranes

Introduction

After recent developments in synthesis and application of nano-baskets as well as analytical instruments, in particular in Mass spectrometry and NMR spectroscopy, the field of metabolite analysis is undergoing fast expansion. Metabolomics is defined as the systematic analysis of large numbers of metabolites to provide major contributions in the fields of toxicology, genomics, *etc*. In the metabolic studies, the accurate levels of metabolites and physiological species in intracellular biofluids are measured. Whether for qualitative or quantitative purposes, the quality and reliability of metabolomics data will depend mainly on the sampling procedures and sample treatment techniques, which receive little attention.

There is no consensus on the adequacy or effectiveness of the available sampling techniques and extraction procedures of metabolites.[1] Owing to low metabolite concentrations and relatively high conversion rates, the common turnover times of metabolic intermediates are in the order of seconds. To avoid unwanted changes in the intracellular metabolite levels, rapidly quench metabolic activity after sampling is already recognized and documented.[2] In the case of cell cultures, the sampling is accomplished by sampling into a cold perchloric acid extraction solution followed by freezing cycles. Although this kind of direct-extraction facilitates the enzymes quenching and releases the intracellular metabolites,[3] however it has two major disadvantages including low biomass levels of metabolites and overestimating, since the metabolites in the extra-cellular biofluids are not removed and lead to the risk of overestimating intracellular pools.

Emulsion liquid membrane (ELM) was invented by Li[4] in 1968 and is known as one of the most promising separation methods for trace extraction of metal contaminants[5] and molecular species[6] owing to the high mass transfer rate, high selectively, low solvent inventory and low equipment cost. Frankenfeld *et al*.[7] reported that ELM could be up to 40% cheaper than other routine solvent extraction methods. This process combines both extraction and stripping stages to perform simultaneous purification and concentration. However, ELM has been limited by the emulsion instability.[8]

Improvement of ELM's surfactants leads to diminish the limitation of emulsions instability. Nano-baskets of calixarenes were introduced as effective emulsion stabilizers. They are a versatile class of macrocycles, which have been subject to extensive researches and extractions,[9] stationary phases,[10] transporters[11] and optical and electrochemical sensors[12] over the past years. Baeyer, in the nineteenth century, synthesized them by reaction of *p*-substituted phenols with formaldehyde in basic or acidic environment.[13] However, the limited analytical instrumental techniques at that time were unable to interpret the structure of the synthesized products. Zinke and Ziegler,[14] in the 1940s, discovered that the products possessed cyclic tetrameric structures. Gutsche,[15] in 1975, introduced the presently accepted name of calixarene. After that, new advances in the field of ion extraction by calixarenes led to introducing new groups such as the ionizable moieties[16] and crown ethers[17] in their scaffolds. The ionizable moieties not only participate in cooperative metal ion complexation, but also eliminate the need to transfer the anions from the aqueous phase into the organic phase by acting in a cation-exchange mode with the metal

cation.[18] Introducing the crown ether ring on the lower-rims, not only increased the cation binding ability of the calixarenic scaffolds but also enhanced their selectivity.[19]

Nano-baskets of calixarenes are a versatile class of macrocycles, which have been subject to extensive researches and extractions (using gas chromatograph, Teif Gostar Faraz Co., Iran), stationary phases, transporters, and optical and electrochemical sensors over the past years.[20-40]

In this study, the ELM process was facilitated by nano-baskets of calixarenes and used as a novel technique for metabolic fingerprinting. For this aim, 12 nano-baskets of calixarenes were synthesized and used as bi-functional surfactant/carrier in the ELMs separations. The maximum metabolic variation was achieved by optimization of calixarene type and concentration, membrane type, stirring rate, treat and phase ratios.

Experimental

The liquid membrane consists of a diluent and a calixarene derivative (as surfactant/extractant). *n*-Decane from Sigma-Aldrich was used as diluent. The experiments carried out using 12 derivatives of calixarene.[9]

The specific amounts of calixarenes were solved in the specific amount of *n*-Decane and thus membrane solutions were prepared. Double distilled water was used as stripping solution. In 10-mL beaker, stripping solution was added dropwise to the stirred membrane solution and the two-phase system was stirred continuously for 30 min at mixing speed of 1500 rpm by a variable speed mixer equipped with a turbine-type Teflon impeller. The mixture of the membrane and the stripping solution was emulsified.

The size, size distribution and stability of emulsions were characterized to examine the method. Size and size distribution of (w/o) droplets obtained by optic microscopy (Mettler FP). The digital format of captured micrographs were analyzed by means of image analyzer software (Digital Micrograph TM, Gatan Inc.). Using a Neubauer camera, the volume of analyzed samples were controlled. By size distribution changes at constant times, the stability of w/o droplets was monitored and evaluated by image analyses from photographs obtained during the diafiltration experiments.

Blood was collected from both male and female rats that had been maintained on different dietary regimens and aged from 12 to 24 months. Blood was collected and the samples were placed on ice for 15 min and then centrifuged for another 15 min. The samples were collected as listed below for individual and pool analyses, placed in vacuum tubes, frozen in N_2-liquid, and stored at −80 ºC before analysis. After that, the samples (200 μL each) were mixed on ice, vortexed, and aliquoted into new tubes (250 μL per tube). Then, each sample (250 μL) was precipitated with 800 μL acetonitrile (0.4%) in glacial acetic acid at −15 ºC, vortexed 20 s, and centrifuged 15 min at −5 ºC. Finally, 800-μL aliquot of the supernatant was collected and stored as the feed solution for the following ELM step.

In 5-mL vial, the ELM prepared was added to some volumes of the above-mentioned feed solution and were stirred by a variable speed mixer equipped with a turbine-type impeller at speed of 500 rpm for extraction time of 30 min. The speed of the mixer was regulated by a voltage regulator. The samples were taken from the stirred cell after the course of the run. The feed phase of the samples was separated from the emulsions by filtration using a filter paper. The emulsions were demulsified by the freezing. Under vacuum, an 800 μL aliquot of the supernatant was evaporated to dryness, then the residues was lyophilized prior to NMR analyses

The lyophilized extracts were re-suspended with sodium phosphate buffer in D_2O (0.1 M) containing TMSP as an internal chemical shift standard. All NMR spectra were measured at 500.11 MHz using Avance spectrometers model DRX-500 (Bruker, CA). The acquisition parameters were a 6-kHz spectral width, 9 μs (60º) pulse, 100 transients collected into 32 k data points, requiring a 9 min total acquisition time and 2.5 s relaxation delay with presaturation of the residual water resonance. Before Fourier transformation, the exponential line-broadenings of 0.5 Hz were applied. The data sets were zero-filled to 64 k points, the spectra were phase and baseline corrected and then calibrated (0.0 ppm, TMSP).

Each spectrum was segmented into 980 chemical shift bars between 0.1 and 9.9 ppm, corresponding to a bar width of 0.01 ppm (5 Hz), using custom-written m-file in MATLAB. The areas within the spectral bars were integrated to yield a 1 × 890 vector containing intensity-based descriptors of the NMR spectrum. The bars located from 4.80 to 5.10 ppm representing the residual water peak and were removed during the calculations. After that, owing to facilitate the comparison between the spectra, total spectral area of the remaining bars was normalized to unity. Then, the data-bars were mean-centered. Principal component analysis (PCA) of the pre-processed NMR data was conducted using the PLS Toolbox within the MATLAB. In this pattern recognition technique, the algorithm calculates the highest amount of correlated variation along the first principal component (PC1), with subsequent PCs containing correspondingly smaller amounts of variance.

Results and Discussion

Type of calixarene is one of the most important factors that influences the selectivity of an inclusion-ELM system, and can often be used in relevant extractions. The effect of calixarene type on the extraction efficiency of low molecular weight metabolites was studied in the ELM process and the spectral results obtained. According to the selected NMR data, there are different spectra for extracted metabolites using calix[4]arenes **1-12** in the ELMs.

The criteria for evaluating the quality of a metabolite extraction method for metabolomics fingerprinting include ease, yield, reproducibility, and speed. Since the metabolites are often co-extracted with other compounds like as lipoproteins and lipids, the net yield of extracts as the total peak

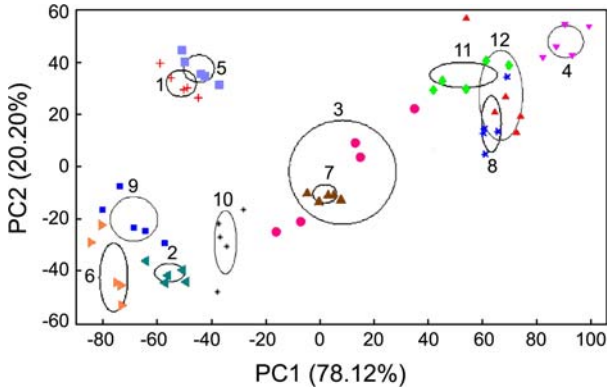

Figure 1. The scores-plots from the NMR spectra using calixarenes **1-12** in the ELMs.

area of the NMR spectra provide an approximate approach for comparing the extraction yields by different calixarene scaffolds. Hence, total spectral area of each extraction was normalized by TMSP signal area and the sample mass, and then the yields were compared between the extractions. Base upon the NMR data, calix[4]arenes **2**, **6**, **9** and **10** extractions produce smaller yields, while the low molecular weight metabolites were generally recovered to greater content by calix[4]arenes **4**, **8**, **11** and **12**. Figure 1 represents the scores plots of NMR spectra and shows distinct differences between the fingerprints from the extractions by different nanobaskets.

Base upon the results, calixarenes **2**, **6**, **9** and **10** tend to extract non-specific macromolecules with high molecular weights, such as lipoproteins and lipids. The corresponding spectra for these materials are presented on the negative side of Figure 1. The ellipses show mean (± SD) for each of extractions. By comparing the extraction procedures for scaffolds **1-12**, it is obvious that the repeatability of **7**-mediated and **3**-mediated ELMs are the most and the least,

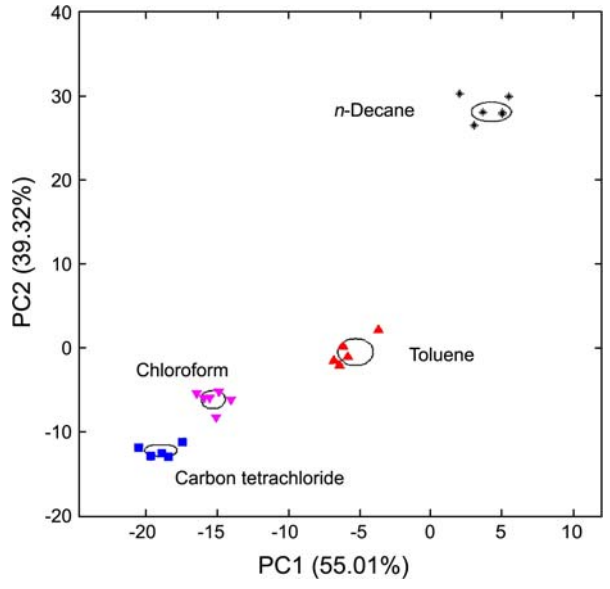

Figure 2. The scores-plots from the NMR spectra.

respectively.

Since the different liquid membranes have different polarities, it was shown that different metabolites were extracted with differing ratios. As mentioned before, total NMR spectral area of each extraction was normalized to sample mass and TMSP signal area in order to compare yields between liquid membranes. According to the NMR data, the yield of extractions using different liquid membranes was in order of carbon tetrachloride < chloroform < toluene < *n*-decane. The effect of membrane type on the extraction efficiency of low molecular weight metabolites were obtained as the spectral results. According to the NMR data, there are four different spectra for extracted metabolites using different membranes of carbon tetrachloride, chloroform, toluene, and *n*-decane in the ELMs.

Figure 2 depicts the scores plots of NMR spectra and distinct differences between the fingerprints from the extractions by four types of membranes.

According to the results, the chlorinated liquid membranes tend to extract the high molecular weight macromolecules. The corresponding spectra for these materials are presented on the negative side of Figure 2. By comparing the metabolic extractions using different membrane types, the repeatability was in order of toluene < *n*-decane < carbon tetrachloride chloroform.

Base upon the peak area, extraction of low molecular weight metabolites increased by increasing the calixarene concentration from 1-5%, while more increase from 5-10% hardly affected the extraction performance. Further increase of calixarene concentration decreased the efficiency of extraction, owing to the access of molecular calixarene in membrane phase. Under the optimum concentration, the molecular form of calixarene is considered enough to forward extraction. Increasing of calixarene concentration up to 5% increased the stability of emulsion liquid membrane, which led to the decrease in the break-up rate; hence, the extraction of low molecular weight metabolites was increased. Further increase in the concentration of calixarene leads to the decrease in the rate of capturing and stripping reaction. This is because the low molecular weight metabolites remain in the membrane without being stripped. This affects the final recovery by the ELM process.

The excessive calixarenes tend to increase the interface's resistance and increase the viscosity of membrane. This increasing from 5% increased the emulsion stability but the mass transfer was adversely decreased. Hence, there is an optimum in the concentration of calixarenes around 4%. The excess of calixarenes concentration leads to osmotic swelling and membrane breakdown. Hence, the concentration of 4% was accepted as optimum concentration. Another criterion is the financial aspects, in which the calixarenes are the most expensive agents among the other components of ELM process, and the lower concentrations are preferred.

The phase ratio is defined as the volume of stripping solution to volume of membrane. At 4:5 phase ratio, the maximum extractions were observed. By increasing the volume of the strip phase, the thickness of film in the

Table 1. The experimental and optimum conditions for the extraction of metabolites

calixarene type	1-**4**	5-8	9-12	-	-
membrane type	CCl_4	***n*-decane**	CH_3Cl	toluene	-
calixarene concentration (wt %)	1	3	**4**	5	10
treat ratio	0.1	0.2	**0.3**	0.4	-
phase ratio	0.4	0.6	**0.8**	1.0	1.2
stirring rate (rpm)	100	200	**300**	400	500

The **bold** items were obtained and used as the optimum conditions

emulsion was reduced owing to dispersion of strip phase in the membrane by mixing. This was favorable in extractions and results in an increase in the extraction of low molecular weight metabolites. Beyond 4:5, the further increase in the volume of strip phase caused the instability of globules.

The treatment ratio, defined as the volume ratio of the emulsion phase to the feed phase, plays an important role in determining the efficiency of ELM process. By increasing the amount of emulsion in the feed phase, the number of available droplets and interfacial surface area per unit volume of the feed solution increases. This leads to increasing the mass transfer of solutes from the feed to the membrane, and more efficiency. Increasing of treat ratio slightly increased the size of emulsion droplets and caused inversely a reduction in interfacial surface area. The increment in the size of droplets was suppressed by the increment in the number of droplets. The results revealed that the extraction efficiency was improved by increasing the treat ratio from 0.1 to 0.3. Beyond 0.3, the further increase in the ratio caused the instability of globules and less extraction efficiency.

The speed of mixing is a key-factor in the rate of mass transfer through emulsion liquid membranes. The effect of stirring speed was investigated in the range of 100-500 rpm in order to obtain optimal speed with effective extraction of low molecular weight metabolites in the ELM process. When the mixing speed was increased from 100 to 300 rpm, an increase in extraction rate was observed. Above 300 rpm the extraction rate again reduced. As a result, an increase in the mixing speed would increase the interfacial area, and this was true up to certain level of mixing speed beyond which an increase in the speed was likely to break the emulsions thereby reducing overall enrichment and the efficiency of extraction. The impact on the wall of a contactor on the emulsion droplets or the shear induced breakage of fragile emulsion droplets near the tip of the impeller imposes upper limit on the speed of agitation. At the same time, swelling was also increased owing to transport of water from feed to strip phase. Some particles are broken owing to shear after reaching larger size. The swollen droplets are breakdown on their own or induced by shear. Therefore, the extraction performance is a trade-off between two effects of swelling phenomena and mixing speed.

The optimum conditions for the extraction of low molecular weight metabolites were determined by the method of one-at-a-time. Table 1 presented all conditions were tested as well as the optimum conditions in bold.

Conclusion

A novel ELM-NMR mediated metabolite extraction method was introduced to minimize the metabolic variation from analysis, and improving the likelihood of metabolic differences and the extraction yield. Considering the yield of extraction and fingerprint, the operational processes of ELMs were optimized and the results are reported in Table 1. Owing to the possibility of selecting different membranes, this method also benefits from extracting the hydrophobic and hydrophilic metabolites into different fractions. Among calixarenes **1-12**, the repeatability of fingerprints for **7**-mediated and **3**-mediated ELMs was maximum and minimum, respectively. Using different membrane types, the fingerprints repeatabilities were determined in order of toluene < *n*-decane < carbon tetrachloride chloroform. Base upon the NMR data, the yield of metabolic extractions followed the order of carbon tetrachloride < chloroform < toluene < *n*-decane.

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