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Metabolic Fingerprinting of Rat Serum by Nano-baskets and ELM – NMR

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A novel approach for metabolite extraction and fingerprinting was introduced base upon the 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 four nano-baskets of calix [4]-1,3-crowns as emulsifier and carrier. The yields of ELMs were optimized by the method of once at a time. According to the 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. According to the NMR data, the results revealed that calixcrowns 1-3 tend to extract non-specific macromolecules and the repeatability of fingerprints for 4-mediated ELM was more than three others. The yield of extractions was obtained to be higher for n-decane and lower for carbon tetrachloride. Using different membrane types, the fingerprints by chlorinated liquid membranes were more repeatable than toluene or n-decane.

Keywords: Nano-baskets, Metabolomics, Calix[4]-1,3-crown, Emulsion Liquid Membranes

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1. INTRODUCTION

After recent developments in 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 led to risk of overestimating intracellular pools.

De Koning and van Dam [4] proposed a sampling method for separating the cells by cold solution of methanol (60 % v/v) and followed by centrifugation, which allowed sub-second arrest enzymatic activity. This method has remained mostly unchanged and is still the most widespread method for rapid sampling of cultures. However, the most critical assumption is remaining the intracellular metabolites inside the cells during quenching and centrifugation. The metabolites leakage from the cells into the methanol solutions may led to underestimating the intracellular levels. Some researchers believe that metabolite leakage did not occur during cold methanol quenching [4], while others reported the occurrence of extensive losses of intracellular metabolites [5].

Emulsion liquid membrane (ELM) was invented by Li [6] in 1968 and is known as one of the most promising separation methods for trace extraction of metal contaminants [7] and molecular species [8] owing to the high mass transfer rate, high selectively, low solvent inventory and low equipment cost. Frankenfeld et al. [9] reported that the ELM could be up to 40 % cheaper than that of other routine solvent extraction methods. This process combines both extraction and stripping stages to perform a simultaneous purification and concentration. Some of the ELM's applications include the separation along with the concentration of sugars, organic acids, amino acids, proteins and antibiotics.

Normally, the feed aqueous phase contains the solute to be extracted. The membrane phase, being immiscible with both the dispersed and the feed phases, separates the two. This phase usually contains additives (carriers) and surfactants too which are chosen in such away that the selectivity, permeability and stability of the membrane layer are enhanced. In a carrier-assisted ELM process, the membrane phase contains the complexing agent of carrier, which has a high affinity towards the solute of interest.

However, ELM has been limited by the emulsion instability [10]. Improvement of ELM's surfactants leads to diminish the limitation of emulsions instabil-

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ity. Nano-baskets of calixarenes were introduced as one of the effective emulsion stabilizers. They are a versatile class of macrocycles, which have been subject to extensive researches and extractions, stationary phases, transporters and optical and electrochemical sensors over the past years. Zinke and Ziegler [11], in the 1940s, discovered that the products possessed cyclic tetrameric structures. Gutsche [12], 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 [13] and crown ethers [14] in their scaffolds. 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.

In this study, the ELM process was facilitated by nano-baskets of calixcrowns and used as a novel technique for metabolic profiling.

2. EXPERIMENTAL

2.1 Chemicals and reagents

The liquid membrane consists of a diluent and a calix [4] crowns derivative (as surfactant/extractant). n-Decane from Sigma-Aldrich was used as diluent. The experiments carried out using four derivatives of calix[4]-1,3-crowns [14].

2.2 ELM Preparation

The specific amounts of calix[4]crowns 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.

2.3 Serum Preparation

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 N2-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.

2.4 Metabolite Extraction by ELMs

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.

2.5 Analytical Instrumentation

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).

2.6 Statistical Analyses

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.

3. RESULTS AND DISCUSSION

3.1 Effect of calix[4]crowns type

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

The criteria for evaluating the quality of a metabolite extraction method for metabolomics profiling 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 area of the NMR spectra provide an approximate approach for comparing the extraction yields by different calix[4]crowns. 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]crowns 1-3 extractions produce smaller yields, while the low molecular weight metabolites were generally recovered to greater content by calix[4]crown 4. Fig. 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, calix[4]crowns 1-3 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 Fig. 1. The ellipses show mean (±SD) for each of extractions. By comparing the extraction procedures, it is obvious that the repeatability of 4-mediated ELM was more than three others.

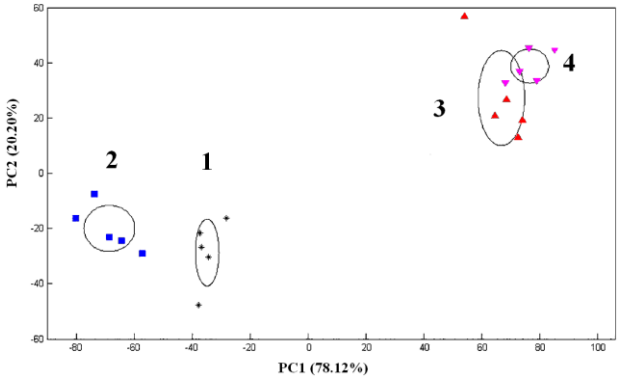

Fig. 1 – The scores-plots from the NMR spectra

3.2 Effect of membrane type

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 was 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.

Fig. 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 Fig 2. By comparing the metabolic extractions using different membrane types, the repeatability was in order of toluene < n-decane < carbon tetrachloride ≈ chloroform.

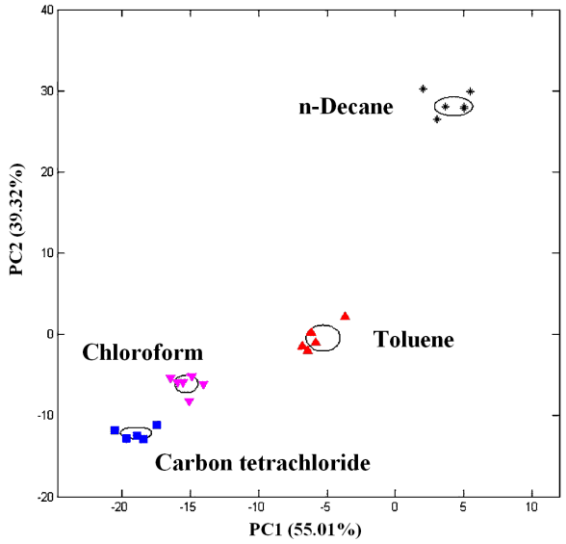

Fig. 2 – The scores-plots from the NMR spectra

3.3 Effect of calix[4]crowns concentration

Base upon the peak area, extraction of low molecular weight metabolites increased by increasing the calix[4]crowns concentration from 1 – 5 %, while more increase from 1 – 5 % hardly affected the extraction performance. Further increase of calix[4]crown concentration decreased the efficiency of extraction, owing to the access of molecular calix[4]crown in membrane phase. Under the optimum concentration, the molecular form of calix[4]crown is considered enough to forward extraction. Increasing of calix[4]crown 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 calix[4]crown 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 calix[4]crowns 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 calix[4]crowns around 4 %. The excess of calix[4]crowns 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 calix[4]crowns are the most expensive agents among the other components of ELM process, and the lower concentrations are preferred.

3.4 Effect of phase ratio

The phase ratio is defined as the volume of stripping solution to volume of membrane. The effect of phase ratio on the extraction of low molecular weight metabolites increased with an increasing of phase ratio up to 4:5. At 4:5 phase ratio, the maximum extractions were observed. By increasing the volume of the strip phase, the thickness of film in the 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.

3.5 Effect of treat ratio

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 depicted that 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.

3.6 Effect of stirring rate

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.

4. 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. Owing to the possibility of selecting different membranes, this method also benefits from extracting the hydrophobic and hydrophilic metabolites into different fractions. The repeatability of fingerprints for 4-mediated ELM was determined to be more than three others. Using different membrane types, the fingerprints repeatabilities were 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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