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Rapid, highly efficient extraction and purification of membrane proteins using a microfluidic continuous-flow based aqueous two-phase system

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ABSTRACT

Membrane proteins play essential roles in regulating various fundamental cellular functions. To investigate membrane proteins, extraction and purification are usually prerequisite steps. Here, we demonstrated a microfluidic aqueous PEG/detergent two-phase system for the purification of membrane proteins from crude cell extract, which replaced the conventional discontinuous agitation method with continuous extraction in laminar flows, resulting in significantly increased extraction speed and efficiency. To evaluate this system, different separation and detection methods were used to identify the purified proteins, such as capillary electrophoresis, SDS-PAGE and nano-HPLC–MS/MS. Swiss-Prot database with Mascot search engine was used to search for membrane proteins from random selected bands of SDS-PAGE. Results indicated that efficient purification of membrane proteins can be achieved within 5–7 s and approximately 90% of the purified proteins were membrane proteins (the highest extraction efficiency reported up to date), including membrane-associated proteins and integral membrane proteins with multiple transmembrane domains. Compared to conventional approaches, this new method had advantages of greater specific surface area, minimal emulsification, reduced sample consumption and analysis time. We expect the developed method to be potentially useful in membrane protein purifications, facilitating the investigation of membrane proteomics.

1. Introduction

Membrane proteins constitute approximately 30% of the proteome [1], playing essential roles in regulating various fundamental cellular functions, such as cell recognition, selective transportation of metabolites and receptor-mediated signal transduction [2,3]. In addition, more than half of the known membrane proteins are predicted to be pharmacological targets [4]. However, researches in membrane proteins are relatively hampered since most membrane proteins are of natural low abundance. Thus, extraction and purification of membrane proteins is usually a prerequisite step in such investigations.

Purification of membrane proteins has proven to be a challenging task due to their hydrophobic nature as complexes of proteins and lipids [5]. Consequently, solubilization of membrane proteins by detergents is necessary to separate them from crude cell extract. Currently, detergent/polymer aqueous two-phase system (ATPS) is a common approach for membrane protein enrichment without denaturation [6–8]. ATPS involves the use of two aqueous phases to extract target molecules by vigorous agitation. Although it has been widely adopted in laboratories, the separation efficiency of ATPS still requires improvement. In addition, emulsification during agitation can also elongate the separation time [9].

The emerging microfluidic technology has provided an opportunity for the integration and miniaturization of existing biological tools to address issues like speed, throughput and sample cost [10–12]. Previously, Kitamori and co-workers reported a microfluidic liquid–liquid extraction system, which was applied to the isolation of metal ions based on multi-phase laminar flows. Extraction of different metal ions was successfully realized, including Fe^{2+}, Co^{2+}, Ni^{2+}, K^{+}, Na^{+} and Al^{3+} [13–15]. The extraction effect of laminar flows in microchannels was equivalent to that of vigorous agitation. However, the microfluidic-based method had advantages of enhanced extraction speed, simplified operation and potential for miniaturization. Recently, Meagher et al. developed a microfluidic aqueous two-phase system (μATPS) for isolating specific proteins from sub-microliter volumes of *Escherichia coli* cell lysate [16]. In this method, PEG-salt two-phase system was realized in a Y-shaped microfluidic channel for continuous extraction of target proteins

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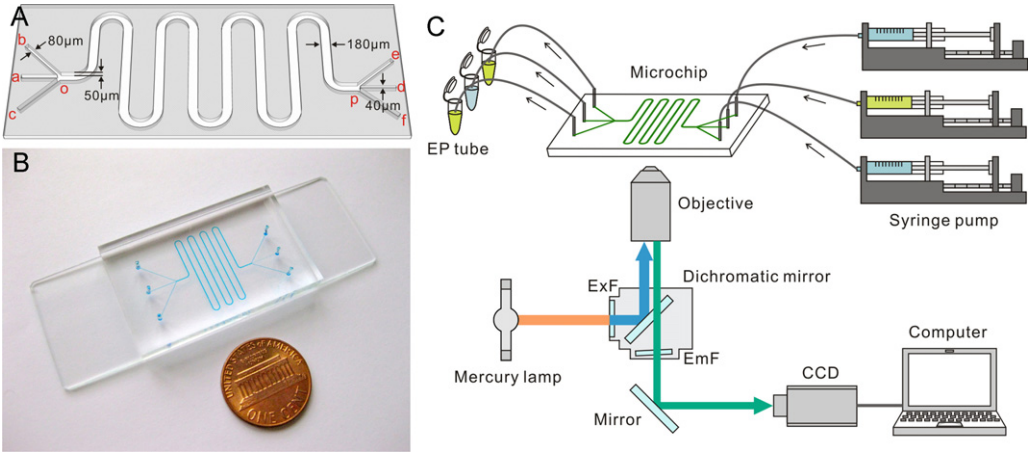

Fig. 1. Microchip fabrication and system setup. (A) Schematic of the microfluidic chip design. (B) Size comparison of the fabricated PDMS microchip with a U.S. one cent coin. (C) Schematic of the system setup for μATPS and image acquisition.

into the PEG phase with reduced separation time, enhanced speed and throughput.

Compared to the traditional batch techniques with agitation, μATPS has distinctive advantages of fast extraction rate, high separation efficiency and sample enrichment [17]. In traditional methods, two liquid phases are highly scattered by vigorous agitation to maximize the specific interface area between the two phases and to improve the extraction rate and efficiency. With microfluidic based method, the laminar flow in microchannels usually results in greater specific interface area, avoiding the use of agitation and preventing the occurrence of emulsification. In addition, liquid–liquid extraction is typically a low-throughput batch technique in laboratory, but it is well-suited for continuous operation on microfluidic chips [18,19]. Therefore, we believe that the μATPS method is potentially useful in the purification of membrane proteins [20,21].

Here, we demonstrated the use of a PEG/detergent μATPS system for the purification of membrane proteins from crude cell extract. Our μATPS system combined the use of the zwitterionic detergent Zwittergent 3-10, sodium dodecyl sulfate (SDS) and the nonionic detergent Triton X-114, resulting in a complementary solubilization of proteins [22,23]. The PEG/detergent two-phase system partitioning allowed successful removal of soluble proteins. Integral and peripheral membrane proteins remained in the detergent phase, while soluble proteins were found in the PEG-rich phase. Extraction of FITC-labeled IgG from detergent to PEG phase was first conducted to evaluate the developed μATPS method. Capillary electrophoresis of the purified samples suggested efficient purification of IgG within 5–7 s. We further applied the μATPS method to the purification of membrane proteins from HeLa cell extracts. Results indicated that 90% of the extracted proteins are membrane proteins, including membrane-associated proteins and integral membrane proteins with multiple transmembrane domains, which represented one of the highest extraction efficiency among existing approaches.

2. Experimental

2.1. Chemicals and reagents

Tris (hydroxymethyl) aminomethane (Tris), HCl, NaOH, KCl, NH_4HCO_3, ACN, NaCl, $NaHCO_3$, KH_2PO_4, $Na_2HPO_4{\cdot}12H_2O$, formic acid, ethylene diamine tetra acetic acid (EDTA) were purchased from Tianjing Chemical Co. Ltd. (Tianjing, China). N,N′-methylene Bisacrylamide, Coomassie Brilliant Blue G250, zwitterionic detergent Zwittergent 3-10 were purchased from Fluka (MO, USA). Dithiothreitol (DTT), iodoacetamide (IAA), acrylamide, glycerol, bromophenol blue, β-mercaptoethanol, polyacrylamide, glycine, polyethylene glycol #6000 (PEG 6000), Trypsin (proteomics sequencing grade) were purchased from Amresco (OH, USA). N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium persulfate (AP), sodium dodecyl sulfate (SDS), Triton X-114 were purchased from Sigma–Aldrich (MO, USA). DMEM were purchased from GIBCO (Invitrogen corporation, USA). Membrane Protein Extraction Kit was purchased from XinHan (Shanghai, China). All reagents were of analytical grade unless specified otherwise. Water was purified by the Millipore-Q system (Millipore, USA) before use for the preparation of all solutions. Samples and all buffer solutions were autoclaved (121 °C; 0.12 MPa) and filtered (0.45 μm microporous membrane filtration) before experiments. For chip experiments, the PEG-rich inlet stream was 35 wt% PEG, and the detergent-rich inlet stream was prepared with a volume ratio of 9:5:1 (20% (w/w) Zwittergent 3-10:100% (w/w) Triton X-114:100 mM SDS), resulting in a pH of approximately 7.4.

2.2. Chip design and fabrication

We designed the PDMS microchip with serpentine microchannels as shown in Fig. 1A. The widths of the inlet channel a–o, b–o, c–o and the outlet channel p–e, p–f are 80 μm. The width of the outlet collection channel p–d is 40 μm. The separation channel o–p has a width of 180 μm and a total length of approximately 140 mm. All channel depths are 50 μm (Fig. 2C). We fabricated the microchip using previously reported protocols [24,25]. Fabricated PDMS structures are then irreversibly bonded to a planar glass substrate (76 mm × 26 mm × 1 mm) to form the final device. A comparison of the microchip with a US one-cent coin is given in Fig. 1B. Micro-syringe pumps are used to control the fluid flow in the microchannels. Typical injection speed was 0.8–1.2 μL min^{-1} of the PEG-rich inlet, and 3.5–5.0 μL min^{-1} of the detergent-rich inlet.

2.3. Image acquisition

Experiments were conducted on an inverted fluorescence microscope (IX 71, Olympus, Japan). A mercury lamp was used as the excitation source. For FITC and FQ, the light emitted from the mercury lamp was filtered by a 460–490 nm band-pass filter, reflected by a 505 nm dichroic mirror, and then focused on the microchannel by a 10× objective (NA 0.7) as illustrated in Fig. 1C. During experiments, fluorescence images of the each local channel were collected through the same objective with a 510 nm high-pass

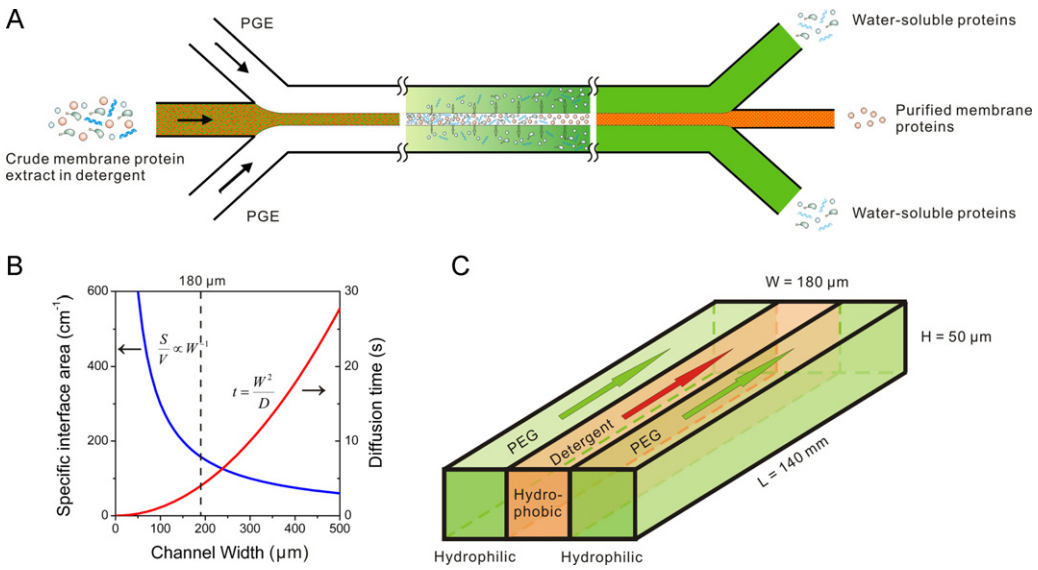

Fig. 2. Extraction of membrane proteins by μATPS. (A) Schematic of the μATPS extraction mechanism. Side streams, PEG-rich phase; middle stream, crude membrane protein extract dissolved in detergent. Black arrows indicate the direction of flow. Hollow arrows indicate the direction of membrane protein migration. (B) Channel width dependence of specific interface area and diffusion time in microchip. *S*, specific interface area; *V*, volume; *W*, diffusion distance; *t*, diffusion time; *D*, diffusion coefficient. Dotted line indicates the microchannel width of our device (180 μm). (C) Profile of extraction channel. *L*, length; *W*, width; *H*, height.

filter and monitored by a CCD camera (CoolSNAP cf2, Photometrics) with 200 ms exposure.

2.4. Cell culture

HeLa cells were grown in DMEM (Invitrogen Corporation, GIBCO, CA) supplemented with 10% NCS (Invitrogen Corporation, GIBCO, CA) and maintained in standard culture conditions (37 °C, 95% humidified air, and 5% CO_2). Cells were allowed to grow to a density of 80% and then were harvested using sterile PBS/EDTA (pH 7.4) before experiments. As they spread out across the cell culture dish, when two adjacent cells touch, this signals them to stop growing, loss of contact inhibition is a classic sign of oncogenic cells.

2.5. Crude membrane protein preparation

Crude membrane protein was prepared by using extraction kit. Briefly, HeLa cells ($(5–10) \times 10^7$) were collected by centrifugation

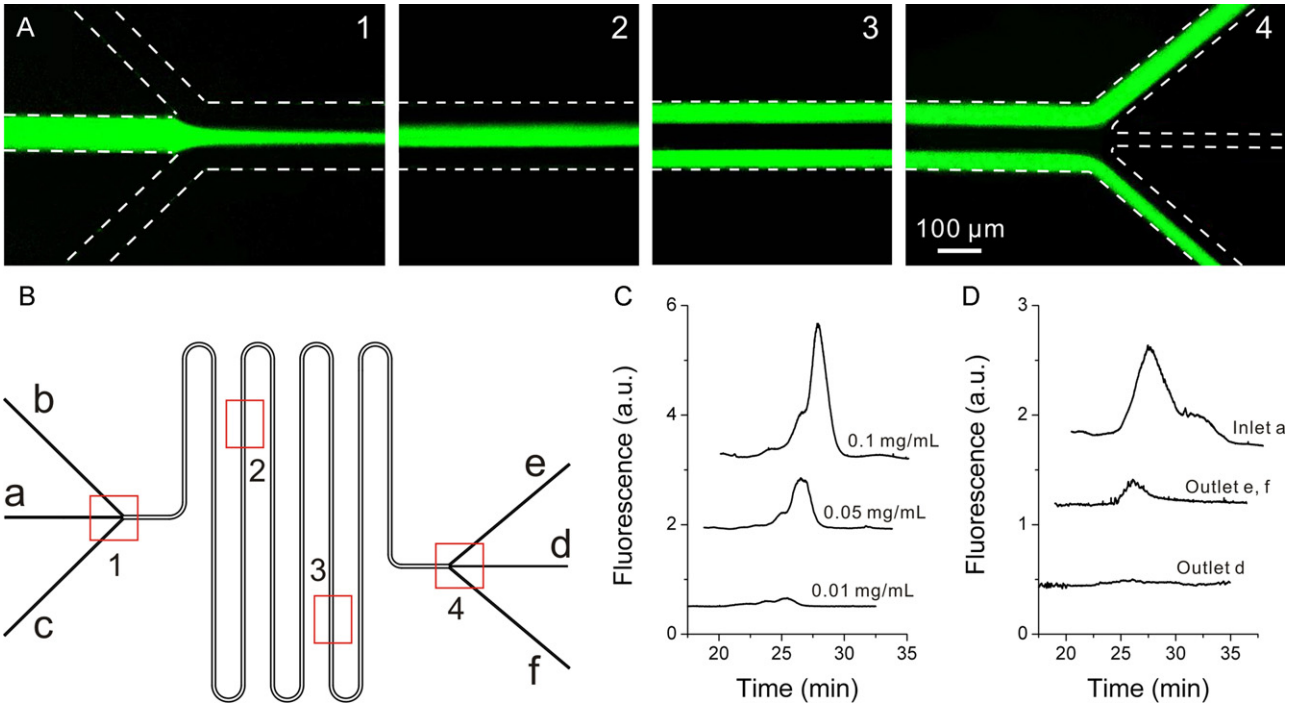

Fig. 3. Evaluation of the μATPS method. (A) Fluorescence images of the extraction of FITC labeled IgG from the detergent phase to the PEG-rich phase. Microchannel outline is indicated by dotted lines. (B) Schematic of the microfluidic chip design. a–c, inlets; e–f, outlets. The red rectangles 1–4 indicate the locations where the four fluorescence images in (A) were captured. (C) Capillary electrophoresis of standard FITC-IgG with concentrations of 0.01 mg mL^{-1}, 0.05 mg mL^{-1} and 0.1 mg mL^{-1}. (D) Capillary electrophoresis of the FITC-IgG in the solutions before and after μATPS purification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

at 1000 × *g* for 5 min at 4 °C, then were resuspended in 1 mL of homogenate buffer with protease inhibitor cocktail in an ice-cold homogenizer and were homogenized on ice for 30–50 times after been washed once with 1 mL of ice cold PBS. The homogenate was centrifuged in 700 × *g* for 10 min at 4 °C. The supernatant was transferred to a new vial and centrifuged at 10,000 × *g* for 30 min at 4 °C, cytosol fraction in the supernatant was collected. The pellet contained proteins from both plasma membrane and cellular organelle membrane. It was resuspended in 200 μL lysis buffer with protease inhibitor cocktail for 30 min at 4 °C, and centrifuged at 10,000 × *g* for 30 min at 4 °C, membrane protein in the supernatant was collected.

2.6. Capillary electrophoresis

Capillary electrophoresis – laser-induced fluorescence detection platform, including capillary, excitation and emission light path, high voltage power supply, optical signal conversion and collection, amplification, filtering devices, signal acquisition programs and hardware, and other related components. The main components include Olympus IX 71 research-oriented inverted fluorescence microscope excitation, emission-axis optical system; high-voltage power supply (Institute of Nuclear Research, Shanghai): the maximum voltage of 30,000 V, maximum current of 0.3 mA; electrode diameter of 0.6 mm of P99 platinum wire; argon ion laser (maximum power 42 mW) as a fluorescence excitation light source; PMT photomultiplier tube and associated circuitry for fluorescence signal collection and amplification; hardware signal filter (Stanford Research System Model SR570); NI 6035 data acquisition card and LabView acquisition program. Uncoated fused-silica capillaries (i.d. 75 μm, o.d. 375 μm) were purchased from Yongnian Optical Fiber Factory (Hebei Province, China) and treated following the protocol introduced by Hjérten [26].

2.7. SDS-PAGE and nano-HPLC–MS/MS

A 200-μg sample of PM protein was separated by SDS-PAGE on a 5% stacking gel and a 12% separation gel run according to standard laboratory procedures. After electrophoresis, the gels were stained with Coomassie Brilliant Blue G250.

In-gel digestion was performed as described previously with slight modification [27]. In brief, random selected bands of SDS-PAGE gel was cut into many 1 mm^3 gel slices, each of which contained a stained protein (s). The resulting slices were washed with 100 mM NH_4HCO_3 containing 50% ACN (pH 8.0) for 3 times, till the dye (Coomassie Brilliant Blue) was completely removed. After being dried in a SpeedVac concentrator, the gel-bound proteins were reduced in 10 mM DTT/50 mM NH_4HCO_3 (pH 8.0) and then alkylated in 55 mM iodoacetamide/50 mM NH_4HCO_3 (pH 8.0) for 30 min in a darkroom. The gel pieces were then washed with 10 mM NH_4HCO_3, and again dehydrated with ACN and dried in a SpeedVac. The dry gel pieces were reswollen with 35 μL of 10 mM NH_4HCO_3 containing 0.5 μg of promega trypsin. Digestion was carried out at 37 °C overnight. The peptides were extracted two times with 40 μL of 60% ACN/5% formic acid by sonication/ultrasonic oscillation/sonic oscillation for 10 min, then centrifuge for 2 min to collect the supernatant. The combined extracts were evaporated to about 2 μL in a SpeedVac and stored at −80 °C.

The digested peptides were injected into a nanoLC system (Eksigent) and first desalted and preconcentrated on a CapTrap (0.5 mm i.d., 2 mm long; MICHROM) precolumn. The peptides were then eluted onto a C18 column (100 μm i.d., 15 cm long; MICHROM) coupled to a quadrupole time-of-flight (Q-TOF) hybrid mass spectrometer (QSTAR ELITE, Applied Biosystems) equipped with a MicroIonSpray ESI source. The gradient profile consisted of a linear gradient from 5% to 40% B (0.1% formic acid/1.9% H_2O/98% acetonitrile, v/v) over 40 min into A (0.1% formic acid/1.9% acetonitrile/98%H_2O, v/v), followed by 10 min of 85% B and then 15 min reconditioning with 95% A. The flow rate was 300 nL min^{-1}. The peptides were detected in the positive ion MS mode or the data-dependent MS/MS mode. The data-dependent mode was used for survey scans (m/z 400–1800) in order to choose up to five most intense precursor ions. For collision-induced dissociation (CID) mass spectrometric (MS/MS) analysis, collision energies were chosen automatically as a function of m/z and charge. The collision gas was nitrogen. The temperature of a heated interface was 150 °C and the electrospray voltage was 2000 V.

3. Results and discussion

3.1. Purification by μATPS

We demonstrate a continuous flow PEG/detergent μATPS. FITC labeled hydrophilic proteins migrated into the PEG phase, while hydrophobic plasma membrane protein from intact membrane protein complexes remained in the detergent phase. This operation is illustrated schematically in Fig. 2A. Sample stream of crude plasma membrane protein with unwanted (tagged) proteins are hydrodynamically focused between two flowing streams containing PEG. The flow-rates of the liquid samples and extracting reagent were controlled by microsyringe pumps (model 210, KD Scientific, Boston, MA) when precise control of the velocity of the sample stream in the main flow channel was required. Each syringe needle was connected to a filter through a fused-silica capillary tube (GL Sciences, 0.25 mm i.d. 0.5 mm o.d.) using epoxy-based glue. The

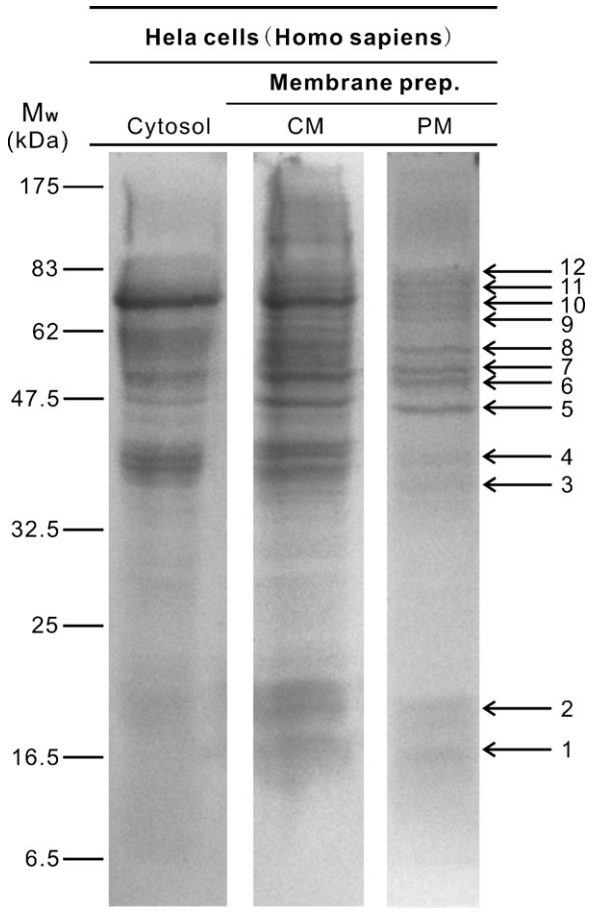

Fig. 4. One-dimensional SDS-PAGE of the prepared crude membrane protein extract. CM, crude membrane proteins; PM, purified membrane proteins. Molecular mass markers are shown on the right. The gel was stained with colloidal Coomassie brilliant blue and 12 bands were randomly selected for further LC–MS/MS analysis.

outlets were also connected to a fused-silica capillary tube with EP centrifuge tube for collection in the same way. For experiments that demonstrate the operations, the velocity of plugs in the main flow channel was ~5 μL min^{-1}. Typically, we found the lower the flow rate, the better the extraction efficiency. However, the laminar flow becomes unstable at too low a flow rate due to the limitation of the syringe pumps. Thus, we chose 5 μL min^{-1} as the optimal flow rate for the extraction of hydrophobic membrane proteins.

3.2. Theory for purification by μATPS

Microfluidics has several characteristic features different from bulk scale fluid flow, such as short diffusion distance, high specific interface area, and small heat capacity. These characteristics of microfluidic systems are essential keys to control chemical unit operations, such as mixing, reaction, extraction and separation. Especially, to control molecular transport in microfluidic channels, the molecular transportation time and the specific interface area must be considered [28]. The molecular transportation time is given by:

$$t = \frac{W^2}{D} \quad (1)$$

where t, W, and D are the molecular transportation time, diffusion distance and coefficient, respectively. The specific interface area, σ, can be expressed as:

$$\sigma = \frac{S}{V} \propto \frac{1}{W} \quad (2)$$

where S and V are the interface area and the volume, respectively.

In our method, samples were injected into the separating channel by flow focusing. For an ideal sandwich-type laminar flow with each stream occupying one third of the channel, the relationship between the microchannel width and the molecular transportation time and the specific interface area is summarized in Fig. 2B. Given the dimensions of our device (Fig. 3C), the specific interface area is approximately 167 cm^{-1}, which represents a dramatic increase compared to the conventional mechanical shaking method (1–10 cm^{-1}) and previously reported Y-shaped microfluidic systems (80 cm^{-1}) [29,30]. In consequence, a significant decrease in the transportation time can be expected. For molecules with a diffusion coefficient of 10^{-9} m^2 s^{-1}, the transportation time is less than 5 s.

3.3. System evaluation

Experiments were conducted with fluorescent tracer molecules to visualize the performance of the μATPS system. Fig. 3A illustrates the fluorescence images captured during experiments with locations indicated by the four rectangles shown in Fig. 3B. As shown in Fig. 3A, detergent phase containing water-soluble FITC-IgG was injected into the middle stream by hydrodynamic focusing. The FITC-IgG was continuously extracted from the detergent-rich stream into the two PEG-rich side streams. Complete extraction of FITC-IgG was observed at the end of the microchannel. Capillary electrophoresis was used to investigate the extraction efficiency of the developed μATPS system. Fig. 3C illustrates a comparison of electropherogram of increasing concentrations of standard FITC-IgG solutions. To determine the recovery of extracted proteins, samples containing 0.05 mg mL^{-1} FITC-IgG were quantitatively analyzed before and after μATPS extraction. As shown in Fig. 3D, FITC-IgG only existed in the solutions collected from the two side streams (outlet e and f), consistent with the optical observations shown in Fig. 3A. Quantitative analysis indicated a recovery of 90.8%. The loss of proteins could have resulted from the nonspecific adsorption of proteins on PDMS surfaces.

Table 1
Categories of purified membrane proteins by μATPS.

Identified membrane proteins	Categories
4F2 cell-surface antigen heavy chain	Plasma membrane
Alkaline phosphatase, placental type	Plasma membrane
Alkaline phosphatase, tissue-nonspecific isozyme	Plasma membrane
Annexin A6	Plasma membrane
Antithrombin-III	Plasma membrane
Calcium-binding mitochondrial carrier protein Aralar2	Plasma membrane
Complement decay-accelerating factor	Plasma membrane
Ezrin	Plasma membrane
Heterogeneous nuclear ribonucleoprotein M	Plasma membrane
Intestinal alkaline phosphatase	Plasma membrane
Junction plakoglobin	Plasma membrane
Lamin-A/C	Plasma membrane
Moesin	Plasma membrane
Olfactory receptor 5AC2	Plasma membrane
Prostaglandin G/H synthase 1	Plasma membrane
Scavenger receptor class B member 1	Plasma membrane
Steryl-sulfatase	Plasma membrane
Transferrin receptor protein 1	Plasma membrane
WD repeat and FYVE domain-containing protein 3	Plasma membrane
78 kDa glucose-regulated protein	Membrane
Carbamoyl-phosphate synthase [ammonia]	Membrane
Carnitine O-palmitoyltransferase 2	Membrane
Cytoskeleton-associated protein 4	Membrane
Glycerol-3-phosphate dehydrogenase	Membrane
GPI transamidase component PIG-S	Membrane
GPI transamidase component PIG-T	Membrane
Lamin-B1	Membrane
NADH-ubiquinone oxidoreductase 75 kDa subunit	Membrane
Nitric oxide synthase, brain	Membrane
Succinate dehydrogenase [ubiquinone] flavoprotein subunit	Membrane
Trifunctional enzyme subunit alpha	Membrane
AFG3-like protein 2	Integral to membrane
Calnexin	Integral to membrane
Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 1	Integral to membrane
Heterogeneous nuclear ribonucleoprotein R	Integral to membrane
Mitochondrial import receptor subunit TOM70	Integral to membrane
Protein disulfide-isomerase A4	endoplasmic reticulum
RNA-binding protein FUS	Nucleus
ATPase family AAA domain-containing protein 3A	Cytoplasm
Elongation factor 1-alpha 1	Cytoplasm

3.4. Extraction of membrane proteins and SDS-PAGE

For membrane proteins, it usually involves a crude membrane protein extraction procedure before further purification and enrichment. Previously, Cao et al. reported the use of conventional aqueous two-phase agitation method for the purification of crude membrane protein extracts, yielding the highest extraction efficiency of 67% [31]. In this work, the developed μATPS system was used in combination with detergents for the purification of crude membrane proteins. After extraction, purified membrane proteins were separated by one-dimensional SDS-PAGE (Fig. 4). Twelve bands were selected for further MS analysis to verify the developed method, similar to approaches reported previously [22,23]. Proteins from the selected bands of SDS-PAGE were digested by trypsin; the tryptic peptides were then extracted from each gel band and further separated by reversed-phase nanoLC, and then detected and sequenced with waters Q-TOF micro mass spectrometer. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot). Mascot was set up to search the SwissProt_57.7 database (selected for Homo sapiens) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 200 ppm.

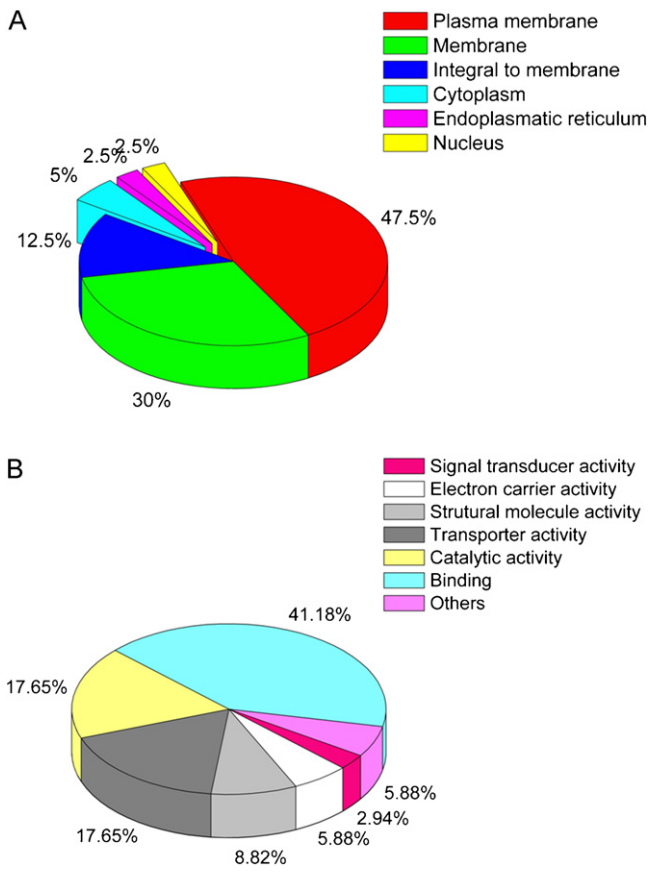

Fig. 5. Classification of the functional categories of the identified plasma membrane proteins in HeLa cells. (A) Subcellular localization of the identified proteins according to the GO annotation terms. (B) The functional categories of the characterized proteins.

3.5. Identification of membrane proteins

After being processed with analytical software Analyst QS 2.0, samples were utilized to search the Swiss-Prot database with Mascot search engine for protein identification. To assess the efficacy of the developed protocol for the enrichment of integral membrane proteins and to estimate contamination by other cellular organelles, including mitochondria and endoplasmic reticulum (ER), we classified the 40 identified proteins according to the gene ontology (GO) annotation and other currently available data (Table 1). Of the annotated proteins, 36 (90%) were previously assigned as integral membrane or membrane-associated proteins. The 90% purity of membrane proteins represented one of the highest extraction efficiency among existing approaches. Of the reminder proteins with a subcellular annotation, 10% were annotated as cytoplasmics, nucleus and ER, this group may include proteins that exist at more than one site in the cell. These data indicate that the contamination by mitochondria and ER in the membrane fraction and soluble non-target protein in cytoplasmics was greatly reduced by use of the microfluidic aqueous two-phase extraction process. In Fig. 5A, of the 36 PM proteins, 19 (47.5%) were plasma membrane, 12 (30%) were membrane proteins, 5 (12.5%) were integral membrane proteins. We also categorized the identified proteins according to their functions, except for 5.56% of the protein function is not clear, based on universal GO annotation terms Fig. 5B: 2.94% have signal activity, 5.88% of proteins have electric carrier activity, 17.65% have catalytic activity, 41.18% are involved in cellular binding, and 8.82% are structural proteins. In addition, 17.65% of proteins are transport proteins which allow the passage of inorganic ions and other small, water-soluble molecules into the cells, 5.88% proteins were not easily categorized and labeled “others”. Since the types of all membrane proteins are still unknown in HeLa cells, we did not compare the number of collected membrane proteins to the total number of membrane proteins. In addition, the loss of proteins during μATPS could not be determined due to unknown number of total proteins and possible loss of proteins during crude membrane protein extraction.

4. Conclusions

In this paper, we demonstrate a microfluidic aqueous PEG/detergent two-phase system for the purification of membrane proteins from crude cell extract. The method was applicable to hydrophobic proteins such as membrane proteins extracted from eukaryotic cells. Our μATPS combined the use of the zwitterionic detergent Zwittergent 3-10, sodium dodecyl sulfate (SDS) and the nonionic detergent Triton X-114, resulting in a complementary solubilization of proteins. The PEG/detergent two-phase system partitioning allowed successful removal of soluble proteins. Integral and peripheral membrane proteins remained in the detergent phase, while soluble proteins were found in the PEG-rich phase. Results indicated that approximately 90% of the purified proteins were membrane proteins, including membrane-associated proteins and integral membrane proteins with multiple transmembrane domains. Compared to conventional approaches, this new method had advantages of greater specific surface area, minimal emulsification, reduced sample consumption and analysis time. We expect the developed method to be potentially useful in membrane protein purifications, facilitating the investigation of membrane proteomics.

Acknowledgements

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References

[1] C.C. Wu, M.J. MacCoss, K.E. Howell, J.R. Yates, Nat. Biotechnol. 21 (2003) 532.
[2] A. Abbott, Nature 426 (2003) 755.
[3] M.C. King, C.P. Lusk, G. Blobel, Nature 442 (2006) 1003.
[4] C.C. Wu, J.R. Yates, Nat. Biotechnol. 21 (2003) 262.
[5] C. Smith, Nat. Methods 2 (2005) 71.
[6] M.W. Qoronfleh, B. Benton, R. Ignacio, B. Kaboord, J. Biomed. Biotechnol. 2003 (2003) 249.
[7] G. Munchow, F. Schonfeld, S. Hardt, K. Graf, Langmuir 24 (2008) 8547.
[8] H. Everberg, R. Peterson, S. Rak, F. Tjerneld, C. Emanuelsson, J. Proteome Res. 5 (2006) 1168.
[9] T. Chapman, Nature 434 (2005) 795.
[10] A. Hibara, M. Tokeshi, K. Uchiyama, H. Hisamoto, T. Kitamori, Anal. Sci. 17 (2001) 89.
[11] T. Minagawa, M. Tokeshi, T. Kitamori, Lab Chip 1 (2001) 72.
[12] K. Sato, M. Tokeshi, T. Sawada, T. Kitamori, Anal. Sci. 16 (2000) 455.
[13] H. Hisamoto, T. Horiuchi, K. Uchiyama, M. Tokeshi, A. Hibara, T. Kitamori, Anal. Chem. 73 (2001) 5551.
[14] M. Surmeian, A. Hibara, M. Slyadnev, K. Uchiyama, H. Hisamoto, T. Kitamori, Anal. Lett. 34 (2001) 1421.
[15] H.B. Kim, K. Ueno, M. Chiba, O. Kogi, N. Kitamura, Anal. Sci. 16 (2000) 871.
[16] R.J. Meagher, Y.K. Light, A.K. Singh, Lab Chip 8 (2008) 527.
[17] G. Munchow, S. Hardt, J.P. Kutter, K.S. Drese, Lab Chip 7 (2007) 98.
[18] J. Atencia, D.J. Beebe, Nature 437 (2005) 648.
[19] P.J.A. Kenis, R.F. Ismagilov, G.M. Whitesides, Science 285 (1999) 83.
[20] J. Blonder, M.B. Goshe, R.J. Moore, L. Pasa-Tolic, C.D. Masselon, M.S. Lipton, R.D. Smith, J. Proteome Res. 1 (2002) 351.
[21] K.K. Hixson, N. Rodriguez, D.G. Camp, E.F. Strittmatter, M.S. Lipton, R.D. Smith, Electrophoresis 23 (2002) 3224.
[22] H. Everberg, T. Leiding, A. Schioth, F. Tjerneld, N. Gustavsson, J. Chromatogr. A 1122 (2006) 35.
[23] H. Everberg, U. Sivars, C. Emanuelsson, C. Persson, A.K. Englund, L. Haneskog, P. Lipniunas, M. Jornten-Karlsson, F. Tjerneld, J. Chromatogr. A 1029 (2004) 113.

[24] Y.N. Xia, G.M. Whitesides, Annu. Rev. Mater. Sci. 28 (1998) 153.
[25] D.C. Duffy, J.C. McDonald, O.J.A. Schueller, G.M. Whitesides, Anal. Chem. 70 (1998) 4974.
[26] S. Hjertén, J. Chromatogr. A 347 (1985) 191.
[27] H. Katayama, T. Tabata, Y. Shihama, T. Sato, Y. Oda, T. Nagasu, Rapid Commun. Mass Spectrom. 18 (2004) 2388.
[28] K. Sato, A. Hibara, M. Tokeshi, H. Hisamoto, T. Kitamori, Anal. Sci. 19 (2003) 15.
[29] M. Tokeshi, T. Minagawa, T. Kitamori, Anal. Chem. 72 (2000) 1711.
[30] M. Tokeshi, T. Minagawa, T. Kitamori, J. Chromatogr. A 894 (2000) 19.
[31] R. Cao, X.W. Li, Z. Liu, X. Peng, W.J. Hu, X.C. Wang, P. Chen, J.Y. Xie, S.P. Liang, J. Proteome Res. 5 (2006) 634.