Supplementary materials

3.Results and discussion

3.1 The development of chip electrophoresis based native protein separationmethod

3.1.1 Comparison of two types of chip electrophoresis channel structure

After IEF, electricity was directly applied at the buffer reservoirs of the second-dimensional channel (C and D) to transfer focused bands from the first channel to the second for further CZE separation.With the criss-cross structure, after protein isoelectric focusing in the first channel, the acid and basic solutions in the first channel reservoirs were replaced with electrophoresis buffer. In the second-dimensional zone, electrophoresis was carried out by applying a positive voltage in pool C and zero voltage in pool D. This type of chip structure was used to realize selective target component transfer by switching the voltage applied between A and D to C and D [1, 2]. However, according to the literature, the focused band was found to be easily destroyed or dispersed during the manual solution-exchange process in the reservoirs. There was intense movement or diffusion of bands due to the change of liquid surface height difference between reservoirs during manual solution replacement. At the same time, it was also not easy to prevent the focused protein band from being pumped out of the channel. The sampling method was then adjusted by first adding10uL acidic and basic solution into the reservoirs A and B, at both ends of the isoelectric focusing channel, respectively. This amount of solution was less than that used previously (10uL vs. 12μL). At the end of the protein isoelectric focusing, a small amount of NaCl (2uL) was filled into the acidic reservoir without pipetting the acidic and alkaline solutions out of the reservoirs. It was found that sometimes the focused protein did not move at all, moved a little, or stayed between the positive and negative reservoirs when electricity was applied. Electro-osmotic flow existing in the untreated channel might also disturb the band transfer. This situation was not like the surface where silanol shielded the channel as reported in the literature. Even though the band was able to move occasionally, the influence of the pressure flow could not be completely overcome. In addition, it was found that with the criss-cross structured microchannel, the focused band could not be easily transferred toward reservoir D alone for CZE separation as expected. More often the band was transfered toward pool B and C on the contrary.

3.1.2 Improvement of sampling

Manual sampling methods with the double T structure chip were studied. Two different ways of sampling were compared, abbreviated as positive pressure method and negative pressure method. The former method was realized under static pressure difference between the different pools, while in the other, an injection was carried out by manual extraction with the help of a syringe pump.

During sampling through manual extraction from B (sample waste) by using a syringe pump, reproducible and precise control of the liquid in the channel was not easily realized. The sample not only filled the whole first-dimension channel but also quickly leaked into the second-dimension channel. The leakage resulted in negative influences on the subsequent protein separation. Therefore, another kind of positive

sampling was utilized taking advantage of static pressure created by the difference in the liquid levels between reservoirs A and B. At first, reservoirs C and D were filled with electrophoresis buffer and reservoir A was filled with protein sample. After the buffer solution in reservoir B was removed, the resulted differences between the liquid surface in reservoir B and A pushed the sample forward from reservoir A to B. The leakage into the secondary channel was inhibited by a relatively higher liquid level in C and D than B. Therefore, with the help of the height difference in the liquid levels, a smooth sample loading without sample leakage was realized. Minor buffer leakage occurred from C and D into the AB channel, but compared with the separation result by the negative sampling method, the improved protein separation demonstrated that the influence of this leakage was minor. At the same time, it could be deduced that the improved positive pressure method was more suitable when the first channel was short. In case of a longer channel, it was found that there was no obvious advantage for the positive sampling method. In the channel longer or narrower with higher capillary force, full injection into the focusing channel purely by difference in liquid levels was difficult. Injection under negative pressure through syringe pump on the other hand would be time saving and easy to control the sampling precisely.

3.1.3 Voltage optimization

3.1.3.1 Effect of voltage during the first isoelectric focusing

The separation voltage used during IEF influenced the performance of 2D electrophoresis separation. The first dimension of IEF was optimized according to the final separation results of two dimension coupled chip electrophoresis, instead of simply according to the IEF result. A higher separation voltage of 900V resulted in a shorter analysis time and better separation resolution. However, if the voltage applied was further increased, the electrophoretic separation was destroyed by the formation of bubbles due to increased heat. Proteins were also in the danger of precipitation. These phenomena were consistent with those reported in other studies [3]. Therefore, use of 900V during IEF was optimal for protein separation.

3.1.3.2 Effect of applied voltage during the second-dimensional electrophoresis

During the second-dimensional electrophoresis, the proteins migrated toward the negative pole, driven by EOF. Separation was optimal at 180V/cm. Compared with 120V/cm, the higher voltage of 180V/cm, applied during the 2nd electrophoresis could reduce sample diffusion. However, if the voltage was as high as 250V/cm, the separation was not improved, together with the influence of the bubbles formed under high voltage was greater.

Using the above optimized electrophoresis conditions, the separation of native standard protein mixtures that contained myoglobin, carbonic anhydrase and catalase by IEF/CZE coupled two-dimensional microfluidic chip electrophoresis was achieved (Appendix Fig.1(2)). The R^2 values of the linearity between the pI value of the protein and its relative position in the channel were all above 0.99 under three voltage conditions. The space between peaks, i.e., the distance between different protein bands, increased with the increase in voltage. Therefore, the excellent separation achieved by IEF, reflected by the good linearity, was retained. Combination of the

two separation modes further improved the separation resolution with increased space between peaks.

3.2 The investigation of biointeraction between α-Syn and small molecules

3.2.1 Voltage optimization

Therefore, the established 2D chip electrophoresis method was used directly in the biointeraction analysis based on the conditions established in the initial part of the study except the voltage. The appropriate voltage during electrophoresis is essential for differentiating between aggregates. For example since IEF focusing promoted protein aggregation, the size distribution of aggregates changed with different IEF voltage. After comparison, it was found that a moderate voltage of 600V during IEF and a voltage of 3000V during 2nd CZE were optimal for study the protein aggregation and the biointeraction between aggregation and drugs.

3.2.1.1 The optimization of voltage during the first isoelectric focusing

In the analysis of α-Syn aggregation in PC12-α lysates, IEF focusing promoted protein aggregation. With increased voltage, the size distribution of aggregates became simpler. Under a high IEF voltage of 900V, only a single mono-dispersed aggregate was observed. Stronger EOF pushed other smaller aggregates into the reservoir. High voltage-induced over-aggregation, in the absence of a neurotoxin, also interfered the investigation on the promotion of α-Syn aggregation by the neurotoxic iron. Few bands were observed and then it was difficult to study the differences between samples.

On the other hand, too low a voltage limited the formation of α-Syn aggregation and impaired observation of inhibition of α-Syn aggregation mediated by drugs either. Therefore, a moderate voltage of 600V was considered optimal for IEF in our biointeraction analysis. Due to the presence of EOF and pH gradient drift, the relative location of the focused band was not only dependent on pI. Therefore, the x-axis was not converted to pH or pI values.

3.2.1.2 The optimization of voltage during second CZE

We also investigated the optimal voltage for the secondary CZE for differentiating between samples. Under IEF, proteins tended to aggregate particularly under higher voltages. When 2D chip electrophoresis was switched from IEF to CZE, the focusing condition and environment changed, and the intermediate focused aggregates were broken up and driven into the 2nd channel. If the voltage was the same as section 3.1.3.2 for pushing small individual protein molecules, the EOF under this voltage was not strong enough to break up and transfer all of the oligomers to the 2nd channel. The differences in the aggregates could not be discerned easily. The information provided by 2Dseparation was limited. Therefore, we applied a higher voltage (3000 V) to drive and transfer larger aggregates.

3.2.2 Sample characterization with traditional biological method

Firstly, the level of α-Syn in α-Syn stably overexpressing PC12 cells (named PC12-α) was determined by western blotting (Appendix Fig.2). Gray-scale analysis revealed higher expression of α-Syn relative to that of β-actin in PC12-α cells. α-Syn protein levels were increased by 20% in PC12-α cells [4]. Furthermore, under the treatment of endogenous neurotoxins N-methyl-(R)-salsolinol, the oligomerates/aggregates of

α-Synwere found in α-Syn over-expressed PC12 cell unlikein non-over-expressed PC12 by in-site fluorescent microscope observation (Appendix Fig.3) [5].

3.3 Study protein aggregation in the hippocampus of mice by chip electrophoresis

3.3.1 Sample characterization with traditional biological method

The feasibility of established chip electrophoresis method into the study of protein aggregation in the sample of animal model was also investigated. First, the protein aggregation in the hippocampus of AD mice was analyzed by immunohistochemical method. After the coronal brain sections were AD from 6 month-old 3xTg-AD and non-transgenic mice were stained with human APP/Aβ-specific 6E10 antibody and developed using DAB, Appendix Fig.4 illustrated that hAPP/Aβ can be readily detected in the hippocampus of 3xTg-AD mice while the section of hippocampus from non-transgenic mice are not immunohistochemically positive for endogenous mouse Aβ. The analysis results were given in Appendix Fig.4.

Reference

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4. Zhang Y, Ma H, Xie B, Han C, Wang C, Qing H, Deng Y (2013) Neurosci Lett 28, 547:65-69.
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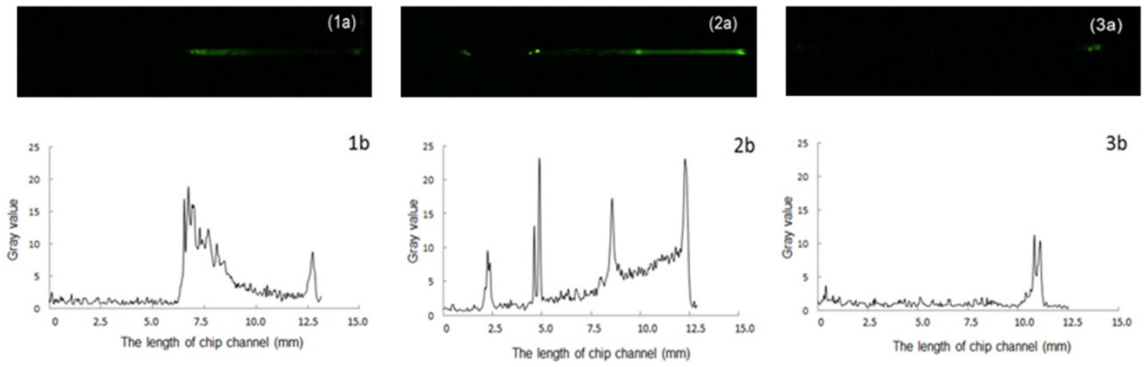
Appendixes

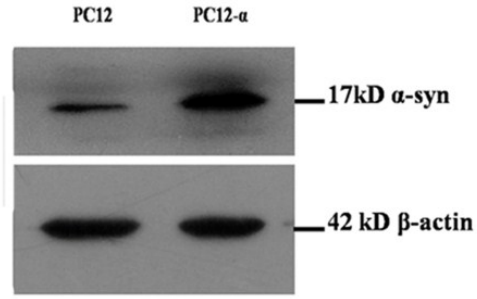
Appendix Table 1

Table 1 The voltage program applied for chip electrophoresis

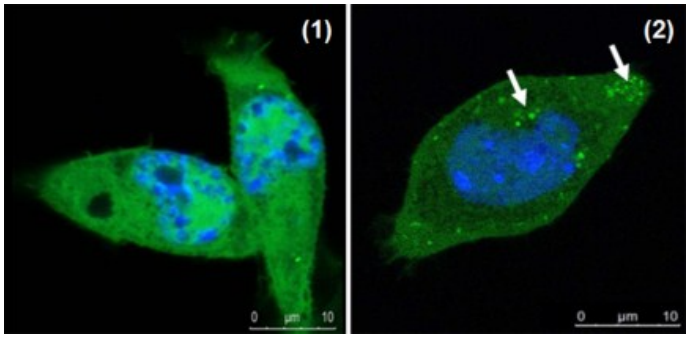
Process (Time, s)	A	B	C	D
Sampling (10s)	Floated	Floated	Floated	Floated
Isoelectric focusing (30 s)	900	0	Floated	Floated
Zone electrophoresis (800 s)	Floated	Floated	2000	0



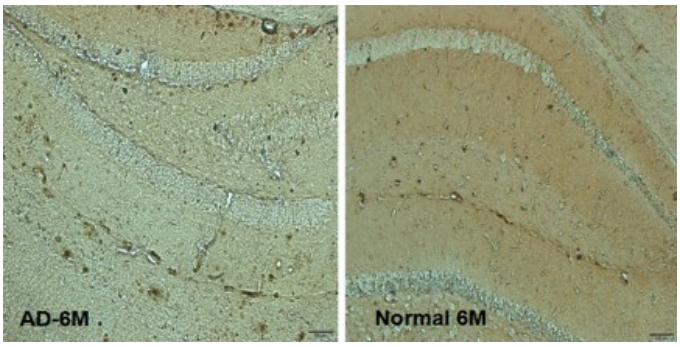
Appendix Fig.1 Isoelectric focusing electropherograms of total protein from PC12 cells overexpressing α-synuclein under different focusing voltages (1)900V; (2) 600V; (3) 300V.



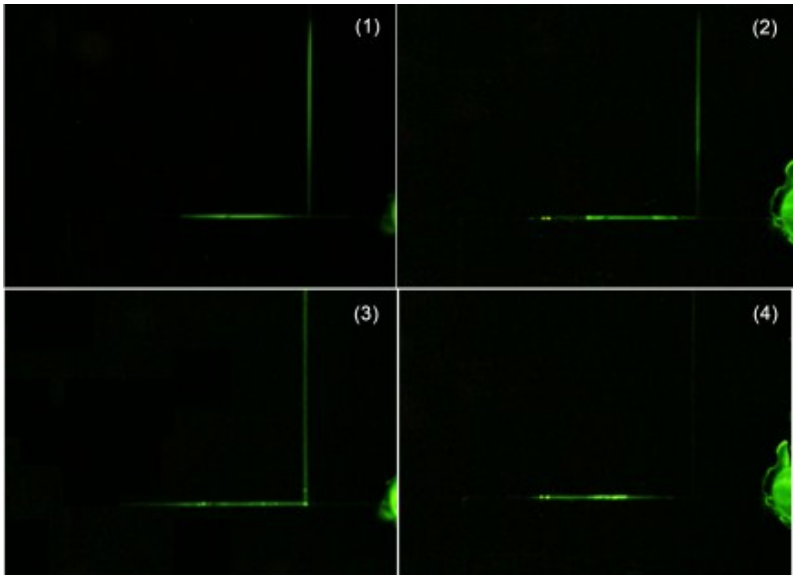
Appendix Fig.2 Expression of α-synuclein (α-Syn) in PC12 and PC12-α cells Western blotting of α-Syn and β-actin



Appendix Fig.3 The in-situ fluorescent microscope image of (1) PC12 cell non-treated with N-Salsolinol; (2) PC12 cell treated with N-Salsolinol



Appendix Fig.4 Immunohistochemical images Image of (1) the hippocampus of non-transgenic mice; (2) of the hippocampus of 3xTg-AD transgenic mice.



Appendix Fig.5 Electropherograms of iron-treated total protein from PC12 cells overexpressing α-synuclein after treatment with rifampicin. (1) A sample treated with rifampicin for 30s, (2) a sample treated with rifampicinfor 90s, (3) a control sample, not treated with rifampicin for 30s, (4) a control sample, without rifampicin treatment for 90s.