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Reciprocating free-flow isoelectric focusing device for preparative separation of proteins

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ABSTRACT

The traditional recycling free-flow isoelectric focusing (RFFIEF) suffered from complex structure, tedious operations and poor extensibility as well as high cost. To address these issues, a novel reciprocating free-flow isoelectric focusing device (ReFFIEF) was developed for proteins or peptides pre-fractionation. In the new device, a reciprocating background flow was for the first time introduced into free flow electrophoresis (FFE) system. The gas cushion injector (GCI) used in the previous continuous free-flow electrophoresis (CFFE) was redesigned for the reciprocating background flow. With the GCI, the reciprocating background flow could be achieved between the GCI, separation chamber and transient self-balance collector (tSBC). In a run, process fluid flowed to and from, forming a stable reciprocating fluid flow in the separation chamber. A pH gradient was created within the separation chamber, and at the same time proteins were focused repeatedly when passing through the chamber under perpendicular electric field. The ReFFIEF procedure was optimized for fractionations of three model proteins, and the optimized method was further used for pre-fractionation of model human serum samples. As compared with the traditional RFFIEF devices developed about 25 years ago, the new ReFFIEF system showed several merits, such as simple design and structure, user-friendly operation and easy to extend as well as low cost.

1. Introduction

Raw protein mixtures obtained from various biological matrices are usually too complex that fractionation prior to analysis becomes crucial processes [1,2]. A number of different separation techniques, including chromatography, selective extraction, protein precipitation and preparative electrophoresis, can be applied to separate proteins and peptides to decrease sample complexities. Among them, free solution isoelectric focusing (FS-IEF) could be used as an effective method for protein sample fractionation.

The FS-IEF shows several advantages: (i) high sample loading up to tens of milligrams [3]; (ii) direct extraction from solution rather than gel matrix; (iii) high sample recovery [3,4]; and (iv) fitting for separation of both hydrophilic proteins and hydrophobic membrane proteins [5]. In the last decades, many attempts have been devoted for the development of FS-IEF techniques. The first kind was Kolin's U-column-based IEF via "artificial" pH gradients [6,7], and Svensson's vertical column stabilized by sucrose density gradient [8]. The second kind was the zone convection IEF introduced by Valmet in 1969, which was composed of a series of linked U-tubes [9]. Several more practical variants based on Valmet's idea were also described latterly [10,11]. But the most popular FS-IEF was multi-compartment device, such as Bio-Rad Rotofor [12–14], Agilent OFFGEL system [15] and Invitrogen's Zoom IEF [16–19].

The last kind was free-low isoelectric focusing (FFIEF). The foremost was recycling free flow isoelectric focusing (RFFIEF) invented by Milan Bier in 1990 [20]. It essentially comprised a focusing cell and an external circulating system. The focusing cell was parallel plate instruments pioneered by Hannig [21]. To drive the process solution cycling through the focusing cell, pumps with a number of channels corresponding to the number of fractions were used. Rapid flow of process fluid successfully counteracted electrically and gravitationally driven convections. RFFIEF has been invented for more than two decades and a commercial instrument has already been made available [22]. Due to the development of free-flow electrophoresis (FFE), continuous free-flow electrophoresis (CFFE) technique was also performed in FFE instruments [23].

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However, RFFIEF had only limited applications [24]. For example, it was used for focusing of amino acids and model proteins [25], proteins extracted from Saccharomyces cerevisiae [26], monoclonal IgG's, single-strand nuclease, and phosphotransferase [27] as well as recombinant human interleukin 10 [23]. Particularly, both RFFIEF and continuous IEF techniques suffered from complex structures, tedious operation, time-consuming and high costs. For example, in RFFIEF it is necessary to use multi-channel pumps with a number of channels corresponding to the number of fractions desired, making the devices more expensive, bulky and complex. Multi-channel pumps and sample collection device made the RFFIEF instrument very complex and expensive [27].

In the previous work, a simple FFE system was developed for bio-separation. Several newly-designed units were integrated into the system, including a gas-cushion injector (GCI) and a gravity-induced self-balance collector (SBC) [28]. Uniform flows of the background buffer in the separation chamber were achieved via the gravity-induced self-balance effect. A multi-channel pump used in traditional FFE device was replaced by the combination of GCI and single channel pump in the novel FFE. Amino acids and proteins were well fractionated in this system [29]. However, the FFE system was designed for continuous free-flow electrophoresis (CFFE), rather than a recycling free-flow isoelectric focusing.

To address the above issues, a novel reciprocating free-flow isoelectric focusing device (ReFFIEF) device with a reciprocating flow was firstly designed in this work. Several units which were used in the CFFE were resigned for the new ReFFIEF system. Then, hydrodynamic environment in separation chamber was investigated and operating procedure was optimized. Finally, the developed method was used for fractionation of model proteins and human serum.

2. Design of ReFFIEF

2.1. ReFFIEF apparatus

Fig. 1A shows the ReFFIEF system, including the separation chamber, GCI, transient self-balance collector (tSBC), fraction collector, power supply, liquid medium pump with single channel, electrode buffer pump and pump controller. The separation chamber was designed based on our previous work [29], comprising the upper polymethyl methacrylate (PMMA) plate, middle spacer and lower cooling plate.

The GCI was a closed box, as shown in Fig. 1A and B, which was constructed with transparent PMMA material. Thirty-two GCI-tubes which had a volume of 5 mL were fixed inside the chamber, leaving 3 cm of the bottom tips outside. Interfaces between the bottom plate of the GCI and the tubes were sealed with PMMA UV glue to avoid liquid leakage. The tips of GCI-tubes were connected with the inlets of separation chamber and the fraction collector tubes by three-way tubes and silicone tubes (Fig. 1A). A multiple soft pipe flow controller (multi-SPFC) described in our previous work [30] was set on the silicone tubes between three-way tubes and fraction collector tubes. The multi-SPFC could close 32 silicone tubes simultaneously if needed. There was an inlet on the bottom of the GCI. The inlet was connected with the liquid medium reservoir (Fig. 1A) via a silicone tube and a liquid medium pump was set between the inlet and the liquid medium reservoir.

As shown in Fig. 1C, 32 inlets were constructed on one end of the upper PMMA plate and 32 outlets were constructed on the other end. Two electrode cells were constructed on both sides of the PMMA plate and two platinum wires were fixed in the cells. Fig. 1D was the photograph of the spacer composed of black silicone membrane and ion-exchange membranes. The black silicone membrane was used for preventing liquid leakage and electric leakage. The anion-exchange membrane (the blue one) and cation-exchange membrane (the yellow one) were respectively used for isolating the cathode chamber and anode chamber from the main separation chamber. The main separation chamber had a net size of 245 mm × 60 mm × 0.4 mm and a total volume of 5.88 mL. A thermoelectric cooling system was used for removing joule heating generated in the separation chamber (not be shown herein) [29].

The tSBC-tubes, just like the SBC-tubes reported in our previous works [28], were connected to the outlets of the separation chamber. Furthermore, two optical water level detectors were installed on one of the outlets and one of the inlets of the separation chamber to monitor the liquid levels. The signals of liquid level changes were conveyed to a pump controller. Then, according to solution level changes, the pump controller would change the running direction of liquid medium pump.

2.2. Working principle of ReFFIEF

Firstly, background solution was fed into tSBC-tubes by hand. Since the way to the fraction collector was closed by the multi-SPFC, solution got trapped in tSBC-tubes, separation chamber and GCI-tubes (Fig. 2A). Then, liquid medium was continually pumped into GCI by liquid medium pump from liquid medium container and an air-cushion was formed in the upper part of GCI. With the rising of liquid medium, the air pressure in GCI increased and the solution in GCI-tubes was pushed out, flowed into the separation chamber and finally flowed into tSBC-tubes. As the solution flowing through the separation chamber, proteins were partially focused under the electrical field (Fig. 2B). When fluid level in the inlet pipes was below the water level detector A (Fig. 2C), signal would be conveyed to the pump controller, the pump controller would change running direction of the medium pump (Fig. 2C).

Then, liquid medium was pumped out of GCI, the air pressure in GCI decreased. Solution flowed back into the GCI-tubes from tSBC-tubes driven by gravity and negative pressure of air (Fig. 2D). Proteins were partially focused again as the solution flowed through the separation chamber. When fluid level in the outlet pipes was below the water level detector B, the running direction of the medium pump would be changed again by the pump controller. Solution flowed back and forth between tSBC-tubes and GCI-tubes through the separation chamber at a speed of 1 mL/min per channel and the proteins were focused repeatedly in electric field until fully focused.

In the newly designed ReFFIEF device, the GCI and tSBC were successfully integrated and made the stable flow achievable. Firstly, the solution level in GCI-tubes always kept at the same horizontal level no matter the background solution was raising, dropping or in steady state due to air-pressure, gratis gravity and principles of connected vessel. Secondly, GCI-tubes had the same shape and were set at the same horizontal level. Therefore, solution flowed into or out at the same flow rate for each GCI-tube when the device was running. Based on the same principle, solution ran into or out of each tSBC-tube also at the same flow rate. This means a certain part of solution just transferred between one GCI-tube and its corresponding tSBC-tube. Theoretically, liquid film in the separation chamber could be seen as dozens of independent streams with no hydrodynamic mixing.

As compared with the traditional RFFIEF, the newly-developed device and technique have the following advantages. First, the developed device showed a very simple structure since the reciprocating flow system was introduced, greatly addressed the inherent limitation of complex design of the traditional RFFIEF [20,22]. Second, the device had highly flexibility. The number of fractions in the new device was not limited by the pump channels any more. Theoretically, any number of fractions could be designed in the novel device just by using the upper PMMA plate with different numbers