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Two-dimensional liquid chromatography consisting of twelve second-dimension columns for comprehensive analysis of intact proteins

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ABSTRACT

A comprehensive two-dimensional liquid chromatography (LCxLC) system consisting of twelve columns in the second dimension was developed for comprehensive analysis of intact proteins in complex biological samples. The system consisted of an ion-exchange column in the first dimension and the twelve reverse-phase columns in the second dimension; all thirteen columns were monolithic and prepared inside 250 µm i.d. capillaries. These columns were assembled together through the use of three valves and an innovative configuration. The effluent from the first dimension was continuously fractionated and sequentially transferred into the twelve second-dimension columns, while the second-dimension separations were carried out in a series of batches (six columns per batch). This LCxLC system was tested first using standard proteins followed by real-world samples from E. coli. Baseline separation was observed for eleven standard proteins and hundreds of peaks were observed for the real-world sample analysis. Two-dimensional liquid chromatography, often considered as an effective tool for mapping proteins, is seen as laborious and time-consuming when configured offline. Our online LCxLC system with increased second-dimension columns promises to provide a solution to overcome these hindrances.

1. Introduction

Two-dimensional liquid chromatography (LCxLC or LC × LC) was first reported by Erni and Frei in the late 1970s [1] and it is now considered a powerful tool in proteomic research [2]. The primary objective toward implementing LCxLC is to improve the resolving power compared to one-dimensional LC (1D-LC). Two-dimensional liquid chromatography can provide a solution for separating and characterizing proteins in complex biological samples and has been an active area of research in the last few decades [3,4].

Theoretical analysis of LCxLC can be traced back to the early 1980s [5,6]. Two important parameters that are frequently considered in the design of LC × LC configurations are "orthogonality" and "throughput". High-degree of orthogonality is usually desired because it allows analytes not resolved in the first dimension (^{1}D) to be separated effectively in the second dimension (^{2}D). In order to obtain high resolving power and peak capacity, distinct separation mechanisms in the two dimensions (i.e. high-degree of orthogonality) are desired [7]. The combination of ion-exchange (IEX) and reverse-phase liquid chromatography (RPLC) are most commonly used due to the increased usage of the 2D separation space [8–10] compared to that of other combinations; i.e. size exclusion chromatography (SEC) × RPLC, RPLC × RPLC and so on [11,12].

Increased throughput is sought when many samples need to be analyzed. Offline LC × LC, where the ^{1}D effluent is fractionated into separate containers and a ^{2}D column chromatographically analyzes each fraction later, has a very low throughput. In addition to this major shortcoming, offline LC × LC is labor-intensive, difficult to automate, and susceptible to sample contamination [4]. In contrast, an online LCxLCLCxLCure [13] may alleviate this problem, a more effective solution is to utilize multiple ^{2}D columns. The first paper using such an approach (two ^{2}D columns) was published by Bushey and Jorgenson [14] in 1997. Since their pioneering work, many alternative approaches have been reported [9,15–19], and a good feature article [2] that summarizes the recent progress of LCxLC has been published. Additional research work and advances on LCxLC have been reviewed by Wang (separation of proteins) [20], Stoll (method optimization and fast comprehensive separations) [21,22], and Jandera (programmed

Abbreviations: LCxLC or LC × LC, two-dimensional liquid chromatography; 1D-LC, one-dimensional liquid chromatography; IEX, ion-exchange; RP, reverse-phase

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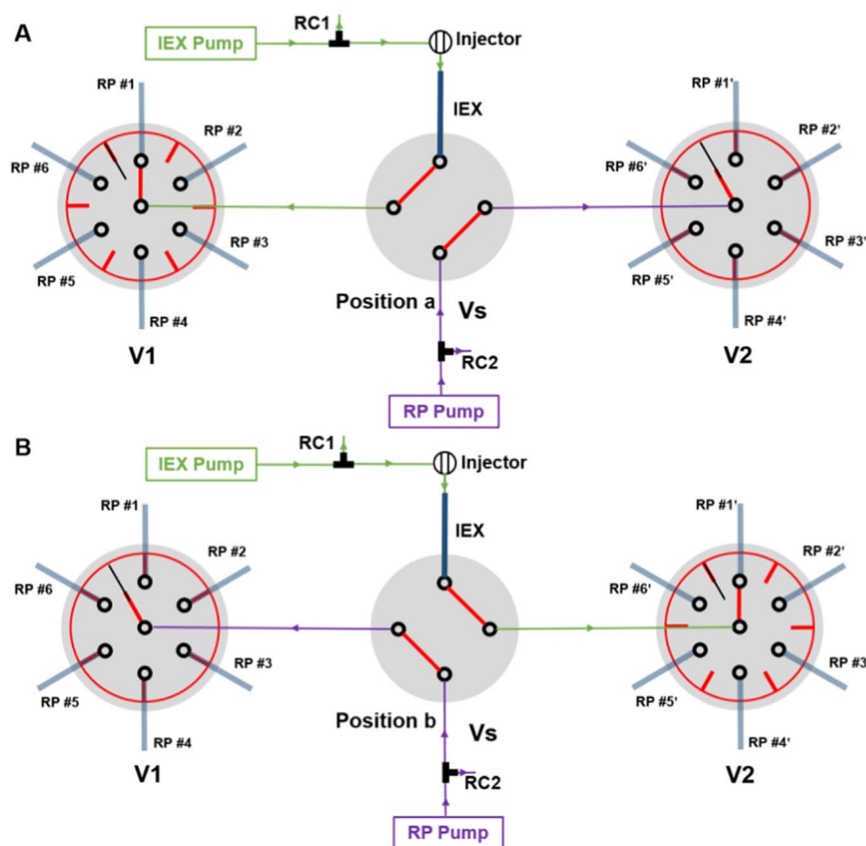

Fig. 1. Schematic description of the online 2D-LC platform with multiple 2nd-D columns. IEX Pump – Dionex GP50, a gradient pump for IEX chromatography; RP Pump – Agilent 1200, a gradient pump for RP chromatography; IEX – monolithic column for IEX chromatography; RP #1, #2, #3, #4, #5, #6, #1′, #2′, #3′, #4′, #5′ and #6′ – twelve parallel monolithic columns for RP chromatography. All the monolithic columns were prepared with capillaries (250 µm i.d.). RC represents restriction capillaries, which were used to control the pressure on columns. Alternate modes of the system configuration are shown in A and B. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

elution) [23].

We have recently reported the use of three ^{2}D columns for LCxLC [24]. To implement a non-stop LCxLC separation, we had to extend the gradient time of the ^{1}D and shorten the gradient time of the ^{2}D with respect to their optimum gradient conditions. We had to inject a 15-min-long segment of the ^{1}D effluent into each ^{2}D column for the ^{2}D separation; some proteins that had resolved in the ^{1}D were mixed again for the ^{2}D separation. To mitigate these issues, here we introduce an innovative configuration (see Fig. 1) that incorporates one ^{1}D column and twelve ^{2}D columns to perform the LCxLC. Using this approach, we can select gradient times closer to their optimum conditions and can inject a much shorter (3.5 min long) segment of the ^{1}D effluent into each ^{2}D column for separation.

2. Experimental

2.1. Reagents and materials

Fused-silica capillaries were purchased from Molex LLC - Polymicro Technologies Inc. (Phoenix, AZ, USA). 3-(Trimethoxysilyl) propyl methacrylate (98%), azobisisobutyronitrile (AIBN), glycidyl methacrylate (GMA, 97%), ethylene glycol dimethacrylate (EDMA, 98%), n-propanol, 1,4-butanediol, tetrahydrofuran (THF), n-decanol, and ten standard proteins (α-Lactalbumen (85%), Cytochrome C (96%), Ribonucleus A (90%), Lysozyme (90%), Transferrin (98%), Myoglobin (90%), Conalbumin (98%), Insulin (27 USP units/mg), Carbonic Anhydrase (95%), and β-Lactoglobulin (90%)) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Styrene (99.5%), divinylbenzene (80%) and diethylamine (99%) were purchased from Alfa Aesar (Ward Hill, MA, USA). Trifluoroacetic acid, methanol, and acetonitrile were obtained from EMD Chemicals, Inc. (Gibbstown, NJ, USA). Tris(hydroxymethyl amino methane) (Tris, 99%), Ethylenediaminetetraacetic acid (EDTA, 99%), and β-mercaptoethanol (99%) were purchased from Thermo Fisher Scientific (Waltham, MA). Deionized (DI) water was purified by a NANO pure infinity ultrapure water system (Barnstead, Newton, WA). Note: Assume HPLC grade for solvents unless otherwise mentioned.

2.2. Preparation of the ^{1}D IEX columns

The ^{1}D IEX monolithic column, poly(GMA-co-EDMA), was prepared. The inner wall of capillaries (250 µm i.d. × 360 µm o.d.) were vinylized with the reported method [25]. The sonicated and degassed polymerization mixture including AIBN (4.3 mg), GMA (225 µL), EDMA, (75 µL), n-propanol (250 µL) and ultrapure water (50 µL) was introduced into a 30 cm vinylized capillary. Then, the capillary ends were sealed with two septa and incubated in the water bath at 60 °C for 16 h. The newly created capillary monolithic column was rinsed with methanol for 4 h followed by ultrapure DI water for 30 min and stored at 4 °C before use. Tertiary amine-modified poly(GMA-co-EDMA) was obtained based on the ring opening reaction of epoxy group by diethylamine [26]. Briefly, a 30 cm poly(GMA-*co*-EDMA) column was placed in an oven at 75 °C and 1 M diethylamine in methanol solution was pumped through the column under a pressure of 500 psi. Two hours later, the amine-modified monolithic column was rinsed with methanol and ultrapure water and stored at 4 °C.

2.3. Preparation of the ^{2}D RP columns

The poly(styrene-*co*-divinylbenzene) monolithic columns were prepared for the ^{2}D separation according to procedures published in the reference [27] with some modification. Four 30 cm vinylized capillaries, as mentioned above, were filled with the degassed polymerization mixture of 4.3 mg AIBN, 50 µL styrene, 50 µL divinylbenzene (DVB, 80%), 130 µL n-decanol, and 20 µL tetrahydrofuran (THF). Then, the septa-sealed capillaries were incubated in the water bath at 60 °C for

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