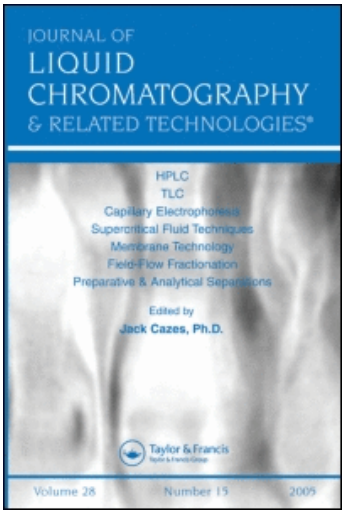

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Yang, Jin-Mei , Chen, Po-Min , Lin, Po-Chun and Chu, I.-Ming(1995) 'Fractionation of Leukemic Cells in Aqueous Two-Phase Systems Using Countercurrent Chromatographic Technique', Journal of Liquid Chromatography & Related Technologies, 18: 9, 1757 — 1766
To link to this Article: DOI: 10.1080/10826079508010004
URL: http://dx.doi.org/10.1080/10826079508010004

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(9), 1757-1766 (1995)

FRACTIONATION OF LEUKEMIC CELLS IN AQUEOUS TWO-PHASE SYSTEMS USING COUNTERCURRENT CHROMATOGRAPHIC TECHNIQUE

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Abstract

Fractionation of various leukemic cells in aqueous two-phase systems, composed of poly(ethylene glycol) and dextran, was performed in a high speed counter-current chromatography (HSCCC) device with eccentric parallel coil orientation. Cells from different lineages or different culture conditions showed different elution patterns. Due to the instability of the stationary phase and the tendency of cells to concentrate at the phase interfaces, special modifications of HSCCC operation were developed to give satisfactory fractionation, these include optimal timing for sample injection, application of downward concentration gradient of mobile phase fluid and proper rotational speed.

Introduction

Cell fractionation is a valuable tool in chracterization of the heterogeneity within a particular cell population, in order to obtain information of medical and diagnostic interests. Distribution pattern of subpopulations is a sensitive indicator for cell differentiation and development

[1]. Centrifugation and elutriation [2] are methods utilize the differences in cell size and density, and are widely used in separating cells of very different properties. Electrophoresis and immuno-ligand based methods [3] are more specific and are suitable for subpopulation fractionation of cells with subtle different properties. Partition in aqueous two-phase systems provides an alternative to look into small differences in cell surface properties in a population [4-6]. The major controling factor for fractionation in aqueous two-phase systems is surface hydrophobicity, as affected mainly by the composition of cell membrane; charges and cell size also affect partition to a lesser extent. Conventionally, counter-current distribution (CCD) was used exclusively for this type of fractionation [7-9]. An alternative to CCD technique is reported here i.e. high speed counter-current chromatography (HSCCC) [10-14]. HSCCC has been used for separating proteins and small molecules and is simpler to operate and easier to maintain sterility than CCD technique. Also, the flow-through type of operation of HSCCC allows connection to many analytical instruments to assist further analysis of cellular properties. However, there remain some problems in adopting HSCCC to this task. Aqueous two-phase systems are much viscous than organic solvents hence phase separation is slow, this results in poor retention of stationary phase fluid. Also, cells tend to collect at phase boundary due to interfacial tension effect [15,16]. This phenomenon is interfering with the partition of cells into two phases, thus, if time is long enough, most of the cells will be located at the interfaces. Hence, normal chromatographic operation will be possible where cells are retained in the columns with no separation or fractionation. A few approaches were employed here to solve these problems. First, a proper coil orientation was chosen. Eccentric columns were found to be able to handle viscous fluid. Secondly, Interfacial surface tension was reduced by using less concentrated systems, tendancy for cells to collect at the interfaces was thus decreased. Further, to enhance the elution of cells from the columns a downward concentration gradient of the mobile phase was applied to further lowering the interfacial tension to free the cells from the interfaces. Thirdly, to obtain good resolution and to prevent settling of cells, rotational speed of the device and flow rate of mobile phase were optimised.

Material and Methods

Poly(ethylene glycol), MW 6000, was from Sigma, USA. Dextran T500 was from Pharmacia, Sweden. Other chemicals, e.g. sodium chloride, were all of reagent grade, and from Merck, Germany. Aqueous two-phase solution was prepared by mixing ingredients thoroughly at room temperature for at least 24 h. Phases were separated by centrifugation at 500 x g for 20 min and stored separately under 4°C, respectively.

A 3-column-in-series type preparative HSCCC was used (CCC-800, Pharma Tech, Baltimore, USA). This seal-less device used triplet columns containings coils of eccentric/parrallel orientation [12]. Coils are made of a single 0.25 cm inside diameter Teflon tube, wound into double-layer coils of 3 cm diameter. The total capacity of the coils is 420 ml.

Cells derived from several leukemic lines were used in this work, see Table 1. Cells were cultivated in T-flasks to late exponential phase. Harvested cells were washed twice in prewarmed buffer and were resuspended in the top phase of the aqueous two-phase fluid, at 10^8 cells/ml. The dextran-rich bottom phase was used as stationary phase, because of its high viscosity. The columns were first filled with stationary fluid. Then PEG-rich mobile phase fluid was pumped in with columns rotating at specified speed. The flow rate is about 1 ml/min. To save time and material, cell-containing sample of 1 to 3 ml was injected via a sample loop 30 to 45 min after the introduction of mobile phase. A fraction collector (Pharmacia, Sweden) was used to collect cells. Cell number and viability was determined under microscopy for each fraction.

Partition coefficient of cells was determined in single test tube partition experiment, where 0.1 ml of cell suspension was mixed with 2 ml of aqueous two-phase fluid and equilibrated for 12 min at room temperature. Phase separation was achieved without centrifugation. Portions of top phase were taken out for cell enumeration. Due to the interfacial concentrating of cells, partition coefficient was defined as the ratio of cell number in the top phase to that of initial cells.

TABLE 1.
Leukemic Cell Lines Studied.

Cell line	HL60	CTV1	RPMI6410	RPMI8402
Origin*	APL	AMoL	ALL	ALL
Differentiation stage	promyelocyte	monoblast	B-blast	T-blast
Ref.	17	18	19	20

* APL: acute promyelocytic leukemia, AMoL: acute monocytic leukemia, ALL: acute lymphoblastic leukemia.

Results and Discussion

A composition of low polymer content for the aqueous two-phase systems was developed. It consisted of (wt/wt): PEG-6000 4.762%, dextran T-500 4.76%, NaCl 0.174%, Na_2HPO_4 0.426% and NaH_2PO_4 0.360%. The phase composition used in this study is a charge sensitive one [4].

The more viscous bottom phase was chosen to be the stationary phase, because a viscous phase is much more difficult to be expelled by a less viscous phase, and thus a higher stationary phase retention. Also, in preliminary runs, the commonly used coaxial type of columns were found unsuitable for this application. This was due to the high mixing action generated by this type of columns and the difficulty in phase separation of the viscous aqueous two-phase fluid. Eccentric type of columns where tube was wound in smaller coils and without the dynamic mixing flow pattern [13] was found more suitable. Stationary retention was found around 10-30% of the total volume of coils. Contrary to the trend observed in coaxial columns [10], higher rotational speeds gave lower retention ratios in eccentric columns, as shown in Table 2. This may be due to larger pressure head needed to be overcome by the mobile fluid in order to flow through stationary fluid pockets under larger centrifugal force field. This resulted in more vigorous mixing and less retention of the stationary fluid. If we use common chromatogra-

TABLE 2.

Stationary Phase Retention in Eccentric Columns with Aqueous Two-Phase Fluid as Affected by Rotational Speed and Flow Rate S.R.: Stationary phase retention, NTP: no. of theoretical plates. NTP was estimated using trypan blue as model compound and using the relationship between half peak width and NTP [21].

	400 rpm		500 rpm		600 rpm	
	S. R.	NTP	S. R.	NTP	S. R.	NTP
1.0 ml/min	33%	236	28%	349	24%	619
1.5 ml/min	33%	172	27%	294	23%	607
2.0 ml/min	31%	164	22%	312	16%	562

phic theory to estimate the number of theoretical plate [21], we found that despite less stationary retention under higher rotational speeds, the number of theoretical plates was higher than those under lower speeds. This can be attributed to the mixing effect mentioned above. Mobile phase flow rate also affects retention and resolution. A rotational speed of 400 rpm and a mobile phase flow rate of 1 ml/min were chosen in order to prevent cell settling on tube wall when centrifugal force is too large and to avoid excessive pressure (up to 100 psi) when flow rate is high.

Mobile phase was introduced to the columns after stationary phase had already filled all three columns. After the mobile phase front reached the exit, small amount of stationary phase fluid, about 7% of the elution solution, came out with the mobile phase. This bleeding of the stationary phase was significant so the timing of sample injection will be important. Elution pattern of cells was found to be greatly affected by this timing. For example, when the sample was injected early, i.e. 15 min after mobile phase introduction to the columns, cells would concentrated at and around

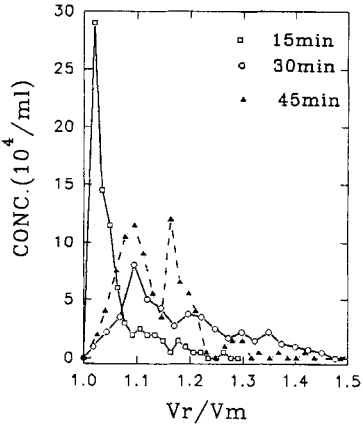

FIGURE 1 Effect of sample injection time relative to mobile phase introduction on the elution pattern of HL-60 cells. Retention volume, Vr, was expressed relative to mobile phase volume, Vm, measured at the moment of sample injection. Only viable cells were counted.

the mobile phase front, and no fractionation was observed. As the sample injection time was delayed, cell elution pattern showed more fractionation, while less cells were eluted (see Fig. 1 for HL-60's results). Some cell lines, e.g. RPMI8402, were found to be totally retained in the columns if sample was injected 45 min after mobile phase introduction (no eluted cell was observed). It is speculated that as mobile phase front moves ahead, it creats a region of unsettling two-phase mixture in its wake where cells do not partition properly. Hence if cells were injected shortly after the front, cells would emerge with the front unfractionated. On the other hand, in region far behind the mobile phase front, the two phases have reached a dynamic balance, the phase boundaries are stable and mixing disturbances are diminishing (this is especially true for the eccentric type columns). Cells bound to the interfaces are therefore difficult to be flushed out by the mobile phase in this region. To obtain fractionation, samples should not be injected too early while application of a downward gradient of the mobile phase should be used to enhance cell elution. Thus

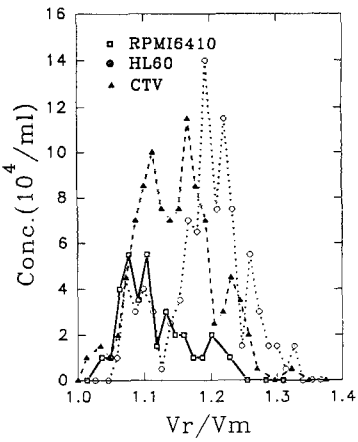

FIGURE 2 Elution patterns of RPMI6410, HL-60 and CTV1 cells. See Fig. 1 for the meaning of Vr and Vm.

cells can be brought out in a gradual, chromatographical mode, when interfacial tension was lowered by the gradient.

As shown in Fig. 2, fractionation of cells was possible using gradient (Fig. 3) method in HSCCC fractionation (only single cell line was used in each run). HL-60 was found to have the most hydrophilic cell surface in the three cell lines tested, since it has the longest retention time. This finding correlated well with the partition coefficients measured by single test tube method. The partition coefficients for the cell lines tested were: 5.43% for Hl-60, 7.50% for CTV1, 8.20% for RPMI6410, and 8.57% for RPMI8402. It is observed that HL-60 has the lowest partition coefficint, i.e. the most hydrophilic one. From Fig. 2, it is noticed that many peaks appeared for a single cell line, signifying certain heterogeneity exists among the population. There seemed to be more of such features (peaks) than observed in CCD, but the signifcance of these peaks are not certain at this time. Also, the recovery of cells was found to vary (data not shown) among different cell lines, further study is needed to clarify this phenomenon.

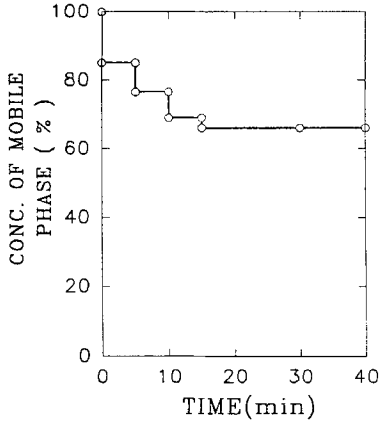

FIGURE 3 The gradient applied to the separation in Figs 2 and 4. Time zero corresponds to the moment of mobile phase introduction. Mobile phase was diluted with buffer solution containing the same amount of salts as in the aqueous two-phase system.

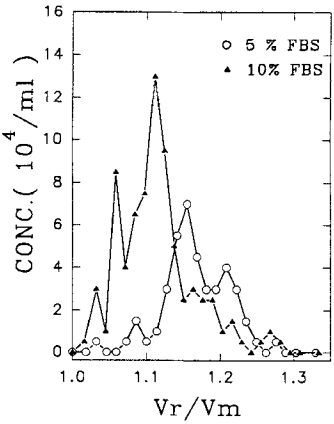

FIGURE 4 Elution patterns of CTV1 cells grown under different FBS levels. See Fig. 1for the meaning of Vr and Vm.

We can readily demonstrate the difference of cell surface properties among cells grown under different levels of fetal bovine serum by this technique. CTV1 cells cultivated under 5% or 10% FBS for 5 days were harvested and fractionated using the gradient method. Cells under 5% FBS were found to be more hydrophilic than those under 10% FBS, see Fig. 4. Cell viability was about the same for these two types of cells, so the different elution patterns reflected shift in cell surface hydrophobicity.

Conclusion

HSCCC was demonstrated to be a potential alternative to the CCD technique commonly employed in the cell fractionation work. Difficulties in handeling aqueous two-phase systems by HSCCC has been addressed. A smaller HSCCC model would be more suitable for cell fractionation since shorter processing time and smaller amount of sample would be required. Further investigation should be worthwhile.

Acknowledgment: This research was supported by Joint Research Grant VGHTH-80-13-02 of Verteran General Hospital and National Tsing Hua University.

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Received: September 13, 1994
Accepted: January 25, 1995