FIELD-FLOW FRACTIONATION OF CHROMOSOMES

Progress Report

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PROGRESS REPORT

ABSTRACT

The work done on this project is divided into two principal areas. The first involves the application of sedimentation/steric FFF to metaphase chromosomes in an attempt to fractionate the chromosomes according to their size. The preparation of chromosomes from a number of organisms was attempted; procedures were finally worked out in collaboration with Los Alamos National Laboratory for the preparation of metaphase chromosomes from Chinese hamster cells. After extensive experimental work was done to identify suitable operating conditions, the partial fractionation of the Chinese hamster chromosomes was achieved.

In the second component of the project, flow FFF was applied to the separation of DNA fragments. Figures are provided that show considerable success in the separation of plasmid digests and in the separation of single from double stranded DNA under 10^4 base pairs. Preliminary work was done on DNA fragments having a size greater than 10^4 base pairs. This work has served to establish the inversion point for DNA.

This progress report provides a summary of work done since the inception of funding, specifically July 1989. The original intent of the work was to use field-flow fractionation (FFF), a powerful family of separation techniques for macromolecules and particles, to isolate and purify narrow subpopulations of chromosomes. Progress along these lines is described under Category 1 below. The scope of the program was later expanded to include the separation of DNA by FFF. The achievements in this area are reported in the second part of the progress report.

1. CHROMOSOME PREPARATION, FRACTIONATION, AND MODELING

Because of our earlier success in the high-resolution separation of various particles in the overall size range $0.5\text{-}50\text{ }\mu\text{m}$ by two different FFF subtechniques--sedimentation/steric FFF [1, 2] and flow/hyperlayer FFF [3-6]--we established as our original goal the separation of chromosomes differing from one another by small size increments. More specifically, we have been able to resolve spherical particles from one another by FFF that differ in diameter by less than 20% in run times of only a few minutes (see Figure 1); it seemed only logical therefore that we could resolve metaphase chromosomes, which tend to be elongated bodies ranging up to $10\text{-}12\text{ }\mu\text{m}$ in length, from one another based on small differences in chromosome length. We therefore believed that a good possibility existed for the near complete resolution of simple sets of chromosomes, but not the resolution of all of the larger complement of chromosomes characteristic of human and many mammalian genomes. Therefore our initial goal was the isolation of metaphase chromosomes from the cells of simpler organisms having low chromosome numbers. We accordingly initiated a program to prepare such chromosomes based on procedures described in the literature [7-13] and advice from numerous experts from this university and other institutions. The preparation procedures were found to be unexpectedly challenging, possibly due to problems of experience and equipment. Thus our attempts to isolate metaphase chromosomes from drosophila, onion, and even human blood cells met with little success despite collaborative assistance and advice from University departments such as Pathology, Biology, and Human Genetics. We elected to proceed to mammalian cells based on new collaborative ties.

Figure 1. Rapid fractionation of polystyrene latex standards by sedimentation/steric FFF. The field strength is 1500 rpm; the flowrate is 6.0 mL/min.

With advice from the Biomedical Sciences Division of Lawrence Livermore National Laboratory and the Life Sciences Division of Los Alamos National Laboratory, we were successful in isolating metaphase chromosomes from the Chinese hamster. On-site instruction as well as the procedure for isolation of chromosomes from the Chinese hamster was provided by Dr. Scott Cram and his group (Los Alamos National Laboratory). Chinese hamster cells (cell line Don from ATCC) were grown in McCoy's medium 5A supplemented with 10% fetal calf serum in a 37°C, 5% CO₂ incubator. The cells were metaphase arrested by adding colcemid to the culture medium to a final concentration of 0.1 µg/mL and incubating for 4 hrs. The cells were then swollen in a hypotonic solution containing 40 mM KCl for 10 to 20 min. Metaphase chromosomes were released from swollen cells by shearing with a syringe needle in isolation solution. Using this method, we have been able to prepare chromosomes of the Chinese hamster cells suspended in isolation buffer for the FFF studies. Increasingly, additional preparations of chromosomes have been provided directly by Los Alamos.

An in-house sedimentation FFF system of the variety typically used for steric mode separations [1, 2] was constructed and characterized using polystyrene latex spheres having diameters corresponding roughly to the size range of the chromosomes. The channel of the system is 86.5 cm in length, 2 cm in breadth, and either 0.0127 or 0.0254 cm in thickness. The channel accumulation wall, after considerable experimentation, was coated with polyimide tape or Teflon to discourage chromosome adsorption. Both a UV and a fluorescence detector (with chromosomes stained by PI or DAPI) were employed for tracking the elution of chromosomes.

Extensive experimentation was directed toward the selection of an appropriate carrier for the chromosome fractionation. In early work, chromosome retention was observed in a Tris-HCl buffer containing Ca and Mg ions as stabilizers. However, collection and microscopic examination of fractions indicated substantial chromosome aggregation and fragmentation. These observations emphasized the need for a carrier buffer which both stabilizes and disperses the chromosomes. The best carrier identified thus far contains KCl, propidium iodide (also a component of the isolation buffer), and Tris-HCl at pH 6.0 to 8.5. Concentrations of individual components may vary but the presence of propidium iodide has been found necessary to observe chromosome retention.

Because the metaphase chromosomes of the Chinese hamster divide into groups with many different lengths (roughly 2-12 µm) [14], we did not expect to isolate any specific length although we hoped to get a fractogram (detector signal versus time) showing at least some partial differentiation into peaks whose content would be further characterized by the collection of fractions and their examination by microscopy. However, the chromosomes eluting from the FFF channel under all the experimental conditions so far attempted appear as a single broad peak with less apparent differentiation than expected. An example of an eluted chromosome peak is shown by the fractogram in Figure 2. The large void peak consists of impurities in the samples including DNA and cell debris.

In order to obtain a more detailed picture of possible chromosome differentiation, fractions of the chromosome peaks were collected and studied by fluorescence microscopy. As we had hoped, the chromosomes eluting earlier were observed to be larger than the later eluting chromosomes, as shown by the micrographs displayed in Figure 2. This elution order confirms the expected operation of the steric mode of FFF. Thus our results show conclusively that we have been able to obtain some degree of chromosome differentiation according to size. While we were disappointed that we were so far not able to obtain a more complete differentiation of chromosomes as could be expected in view of Figure 1, the partial differentiation shown in Figure 2 combined with the removal of many interfering impurities is extremely promising in providing a partially purified feed material for flow cytometry. The importance of this partial purification (or enrichment) is described in the body of the research proposal; its further implementation constitutes

Figure 2. Fractionation of Chinese hamster chromosomes in a sedimentation FFF channel with fluorescence micrographs of collected fractions. FFF conditions: field strength = 600 rpm; flowrate = 1.23 mL/min; carrier of pH 8.5 is 10 mM in Tris-HCl, 75 mM in KCl, and contains 25 μ M/mL propidium iodide.

the lead project of future proposed work.

We have also expended considerable effort in applying flow FFF (as distinguished from sedimentation FFF, which was used in the experiments described above) to the separation of chromosomes. The results appear to be roughly comparable to those obtained using sedimentation FFF.

Although our results, from a practical point of view, are extremely promising, we have proceeded with further research to determine why we did not get a higher level of chromosome differentiation as expected based on many previous FFF experiments such as shown in Figure 1. At the same time we wished to gather more evidence on another anomaly, specifically the fact that the retention time is much shorter than expected based on the reported density (~ 1.3 g/mL) [15, 16] and the observed size of the chromosomes. In order to shed light on these unexpected results and through this more complete understanding improve chromosome resolution, we have undertaken a program involving the study of model particles.

Many metaphase chromosomes are roughly cylinder-shaped (or capsule-shaped) with a cylinder length greater than the diameter. Within a species, it is common to find chromosomes of fairly uniform diameter, differing mainly in length. Basic field-flow fractionation concepts [2, 3] suggest that particles of the shape mentioned can be separated by virtue of both the differential driving forces of FFF and the unequal hydrodynamic lift forces operative in the steric mode. However, while the resolution of spherical particles on this basis is well understood and efficient, there is no explicit theory and no systematic experimental results that predict retention and resolution levels for nonspherical particles like chromosomes having dimensions ≥ 1 μm . (The retention of submicron nonspherical particles is readily predicted based solely on particle mass [17].

To confirm the qualitative prediction that separation should be reasonably efficient for elongated particles, we have shown that it is possible to fractionate doublet aggregates of 2 μm polystyrene latex beads from singlets. (Larger clumps were even more cleanly separated.) These two populations closely model different Chinese hamster chromosomes in size and shape. The identification of the latex peaks was confirmed by electron microscopy.

Glass fibers constitute another class of materials that can be used as a model for the cylindrical chromosomes. Preliminary studies have been carried out using fibers ground to a distribution of short (chromosome-like) lengths. Of particular interest is the fact that sedimentation FFF data on the glass fibers assign them to an effective size range (by comparison with polystyrene standards) significantly smaller than their length but greater than their diameter. However, the length-based selectivity was reasonably large, 0.6 to 0.9 as compared to ~ 0.8 for spheres. Thus a strong separative effect is shown to exist for particles of this shape.

Using a theoretical treatment developed by Jeffery [18, 19], calculations on glass fibers were carried out to determine the relative translational and rotational motions of the particles in liquid suspension subjected to a velocity gradient. It appears that longer cylinders spend most of their time aligned with the flow while shorter cylinders spend more time at other angles relative to the flow axis. In the real environment of the FFF channel, the external field will depress the rotation of the rod shaped particles because the tumbling of the rod requires overcoming the external force. These phenomena would be detrimental to steric mode fractionation were it not for the influence of the hydrodynamic lift force which is dependent on particle size. Clearly, these studies must be extended to understand the origins of resolution (which is found to be satisfactory) for these rigid rods and then to draw the necessary parallels with chromosomes so that their resolution can be improved.

2. DNA SEPARATION

In view of the importance of isolating DNA fragments for genome work, we have initiated a program to examine the applicability of FFF in this area. Since DNA fragments in the range 10^2 - 10^4 base pairs and beyond are frequently required, our program scope was chosen to cover a very broad range of these molecular sizes. In planning to cover such a broad range, it is necessary to realize that there is a fundamental transition between FFF retention mechanisms at about 10^4 base pairs, the exact chain length depending on flowrate and other conditions. This transition is associated with an inversion in the elution order [3, 20] as described earlier. Thus the DNA separation program was divided into two parts, involving DNA chains under and above 10^4 bp, respectively.

To this point, we have focused primarily (but not exclusively) on the separation of the lower molecular weight DNAs. This option was chosen because the mechanism for separating these shorter polymer chains is well understood based on our previous work with numerous synthetic polymers and biopolymers [21, 22]. For the shorter chain lengths, separation takes place by virtue of the balance between external driving forces and diffusion, as characteristic of the so-called normal mode of FFF operation [23].

For applications to DNA we have used flow FFF, which is not only capable of fractionating materials of much smaller size than sedimentation FFF, but is applicable at very high molecular weights as well. Our first goal was simply that of determining whether DNA fragments could be retained and separated in flow FFF channels having different primary inlet configurations. More particularly, numerous DNA samples have been studied with three different inlet configurations: a normal triangular inlet, a frit inlet, and a split inlet. With the latter two, the stopflow procedure can be bypassed, thus streamlining operation [24].

For shorter ($<10^4$ bp), we have had considerable success using both frit inlet [24] and normal triangular configurations. However, the best resolution has been achieved with the triangular configuration (necessitating the use of the stopflow procedure), for which good initial results have been obtained. Several of the separations are shown by the fractograms in Figures 3 and 4. These figures show that flow FFF has already advanced to the point of being highly successful in the separation of plasmid digests and the easy resolution of single and double stranded DNA. Most of our results have been confirmed by applying gel electrophoresis to the collected fractions as shown in Figure 3.

For the separation of double from single strands (Figure 4), the chain stiffness of the double strands causes them to exhibit a lower diffusion coefficient than the single strands of the same contour length. Thus arises the differential retention in flow FFF, leading to a baseline resolution for these forms of DNA plasmids within ~20 minutes.

While we have achieved favorable separations in this study as shown by the figures, we have not yet fully optimized the separation. Our work to this point has been more fully focused on understanding the mechanism of the separation and in examining the agreement of the experimental results and theory. We have had good success in this correlation.

Although stopless flow injection is preferable to stopflow in order to eliminate the stopflow procedure, zone broadening remains a concern for the former. A study of flow conditions using plasmid DNA samples in the frit-inlet stopless flow channel indicated that a minimum plate height was obtained with the use of a high (97/3) value for the ratio of the frit inlet flowrate to the sample inlet flowrate. While the conditions mentioned resulted in the minimum plate height for the stopless flow procedure, the stopflow procedure produced still narrower peaks.

Figure 3. (a) Separation of DNA fragments by flow FFF with gel electrophoresis of collected peaks. Injection sample: 0.35 μ g pBR322 digested by *Hinc* II resulting in two fragments 3.25 kb and 1.11 kb. Electrophoresis data: lane 1 - DNA marker; lane 2 - pBR322 digested by *Hinc* II; lane 3 - fragment 1.11 kb recovered from peak 1; lane 4 - fragment 3.25 kb recovered from peak 2. Flow conditions: crossflow = 1.05 mL/min; stopflow time = 2.0 min. Channel flowrate = 3.15 mL/min. (b) Separation of DNA fragments as shown with channel flowrate = 6.70 mL/min yielding more rapid fractionation without loss of resolution.

Figure 4. Separation of two different sets of single strand circular DNA and double strand plasmid DNA in a flow FFF channel of thickness 0.0230 cm. FFF conditions: cross flowrate = 1.08 mL/min, channel flowrate = 3.15 mL/min, carrier Tris-HNO₃ of pH 7.8.

In normal mode flow FFF, sample components are eluted in order of increasing molecular weight while in the steric (or hyperlayer) mode, higher molecular weight species emerge from the channel earlier [2, 3, 20]. The steric transition point (the point of reversal in FFF elution order) was found experimentally to correspond to a DNA molecular weight of 5 to 6 million daltons for a given set of flow conditions. Theoretical treatments based on the radius of gyration and the diffusivity calculated from the Mandelkern-Flory theory [25] predicted steric inversion at approximately 9 million daltons, in reasonable agreement with our observations. The discrepancy suggests the presence of a lift force effect. Understanding of the lift force phenomenon has been an ongoing priority in our theoretical and experimental work [3, 20].

The lift force effect for high molecular weight species migrating in the steric/ hyperlayer mode has generally been considered of hydrodynamic origin. As an alternate approach, an attempt to correlate FFF data with the predictions of an entropic lift treatment (the stress-induced diffusion theory of Tirrell and Malone) was undertaken [26]. The latter theory was found to greatly overestimate the lift force as well as the dependence of the retention ratio on DNA molecular weight.

Finally, FFF data can be used to calculate physicochemical properties of the DNA species which relate to molecular structure. Comparison of the flow FFF-determined diffusion coefficients with the diffusion coefficients predicted by various theories of polymer chain structure have been made. DNA species of increasing molecular weight could be progressively described as rod shaped (Tirado-Garcia de la Torre theory [27]), wormlike chains (Yamakawa-Fujii theory [28]) and random coil chains (Kirkwood-Riseman theory [29, 30] as well as those mentioned above). The agreement of the theoretical values predicted by these theories and the experimental values is very good, confirming the expected mechanism of FFF separation.

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