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Journal of Chromatography B, 664 (1995) 444–448

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Selective elution and purification of living *Trichomonas vaginalis* using gravitational field-flow fractionation

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First received 26 August 1994; revised manuscript received 14 October 1994

Abstract

Gravitational field-flow fractionation is one of the simplest separation methods for biological materials. Its potential in parasitology is demonstrated for *Trichomonas vaginalis*, a parasite responsible for one of the most widespread sexually transmitted diseases. It was observed that this unicellular parasite can be purified in a culture medium with a recovery of 85% for the living trophozoites. The parasite retention characteristics were different when motile living and non-motile dead cells were eluted, motile cells being less retained than the non-motile cells.

1. Introduction

Field-flow fractionation (FFF) was initiated by Giddings [1] in the early 1970s and its elution principles have been widely described. In its simplest form, gravitational FFF (GFFF), a carrier phase flows in a horizontal ribbon-like channel at the inlet of which samples can be injected. This simple FFF device, which uses the earth's gravity as a transverse field, has shown great potential in the separation and purification of cellular material of different origins, including multicellular blood parasites such as microfilariae [2–4].

Micrometre-sized particles such as cells are eluted following an elution mode described as "steric hyperlayer" [1] and, at the outlet of the channel, eluted fractions can be collected and detected. Non-motile cells of different origin have been widely analysed and purified using this method [2,5–8]. It has also been demonstrated that when motile *Escherichia coli* were eluted in FFF, the retention properties varied according to the motility of the bacterial species [1]. Such results indicated the feasibility of using FFF for the study of parasites in terms of diagnosis and pharmacology.

The protozoan flagellae *Trichomonas vaginalis* is responsible for human trichomoniasis, one of the most widespread sexually transmitted diseases (STD). Several methods are currently used for laboratory diagnosis, including direct microscopic observation, cell culture and immunological techniques. The parasites in wet mounts move with a nervous and jerky motion via four anterior flagellae and an undulating membrane.

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0378-4347/95/$09.50
SSDI 0378-4347(94)00498-6

We have used the very simple separation principle provided by GFFF to study the elution of *Trichomonas vaginalis*, both for the motile living and the non-motile dead trophozoites.

2. Experimental

GFFF separation devices have already been described [9], in particular for biological applications [2,5,10]. In this work, a system analogous to that employed for red blood cell elutions was used with the dimensions: $49.5 \times 2 \times 0.0175$ cm. Inlet and outlet tubings (Upchurch Scientific, Oak Harbour, CA, USA), 20 cm × 0.0256 cm I.D., were both glued on the accumulation wall. The inlet was connected to a Rheodyne (Cotati, CA, USA) Model 7525 injection device with a 20-μl loop and the outlet was fed to a Knauer (Strasbourg, France) variable-wavelength detector set at 254 nm. A Gilson (Champigneulles, France) Model 302 chromatographic pump was used. Parasite suspensions were made with the carrier phase [isotonic phosphate buffer, 0.1% (w/w) albumin] and injected into the established flow without relaxation. The average flow-rate was set at 0.33 ml/min.

Trichomonas vaginalis strain CMP (Châtenay Malabry Parasitologie) was isolated from a woman suffering from STD in 1987 and stored as stabilate in liquid nitrogen containing 6% dimethyl sulphoxide as cryoprotectant. This strain was metronidazole-sensitive. The culture was performed in TYM medium [11] with 10% filtered horse serum (Institut Pasteur, Cat. No. 61311) added. Culture tubes (5 ml) were inoculated with 10 000 parasites/ml and incubated at 35°C; the sub-cultivation frequency was 2 days. Parasites reached an average number of $(1–2) \cdot 10^6$/ml 48 h after inoculation and appropriate suspensions were performed in the carrier liquid prior to injection. The parasite average size was determined microscopically on wet mounts (×1000). Approximately 50 000 parasites were injected through the Rheodyne injection valve into the channel. The viability of the parasites was checked microscopically: living parasites are motile and dead parasites are non-motile. To enhance the photometric detection of the parasites, in some experiments living staining was performed with propidium iodide (200 μg/ml). The signal was monitored during elutions and systematic fraction collection in 1-ml Beckman tubes was time controlled manually. The quantification of the parasites in the collected fractions was performed after centrifugation (200 *g* for 5 min) and concentration to 50 μl. The number of parasites in each tube was determined microscopically using an haematocytometer (Kova Slide 10; Boehringer-Bacteriologie). Parasites were killed when necessary using a 10% (v/v) formalin solution and washed with the carrier phase immediately before injection. Neither the shape nor trophozoite density was modified using this procedure. The formalin solution was prepared by mixing 37% (w/w) of formaldehyde with water and dilutions of formalin were made with isotonic phosphate buffer.

3. Results and discussion

3.1. Photometric detection of Trichomonas vaginalis

Injection of the living parasites without specific treatment did not provide any significant photometric signal at the outlet of the channel, although fraction collections showed a significant amount of motile trophozoites. Therefore, to enhance photometric detection, living staining was performed using propidium iodide and the characteristic profile of a fractogram was obtained, as shown in Fig. 1. When recorded signals were needed, staining was performed before injection.

Systematic elution of stained parasites at constant flow-rate showed good reproducibility with a retention factor of 0.606 ± 0.05 (2σ, $n = 4$). The recovery was also reproducible, reaching 85% for the living and 55% for non-motile trophozoites. The incomplete recovery suggests that possible irreversible particle–wall interactions could occur. The high yield of living material allowed for quantitative measurements on

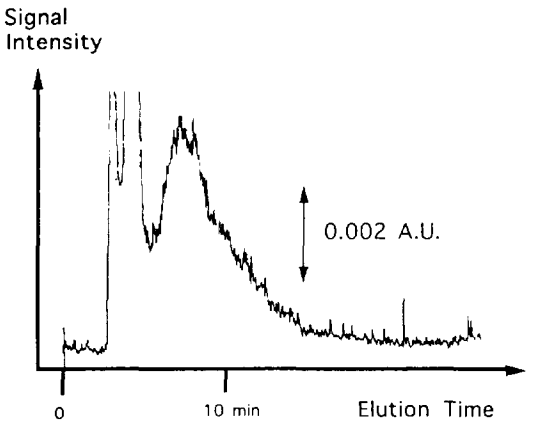

Fig. 1. Stained *Trichomonas vaginalis* fractogram. Sample suspensions were injected into the flow through a Model 7525 Rheodyne device. The channel void volume was 1.73 ml, the elution flow-rate was 0.331 ml/min and ca. 50 000 parasites were injected in a 20-μl volume. The detection wavelength for propidium iodide-stained parasites was 254 nm. Three characteristic peaks are observed on the factogram, the most retained corresponds to *Trichomonas vaginalis*. The origin of other eluted peaks has been described previously [2,3].

collected motile parasites, although the accuracy was low for the non-motile parasites.

The low recovery observed for non-motile parasites suggests that the polycarbonate used for the experiments described here could be replaced with other materials giving weaker particle–wall interactions [3,5]. However, no systematic studies are yet available to suggest clearly how to manage the parasite–wall adhesion mechanism. Therefore, the yield values observed for this series of experiments were interesting enough in terms of quantitative evaluations. The observed yields were lowered using the stopped-flow relaxation time [1], so injections into the established flow were performed.

3.2. Separation of motile and non-motile parasites

A very simple methodology was used to compare the elution patterns of living and dead parasites, in other words to analyse the retention differences due to parasite motility. As staining procedures may modify their physiology and decrease the parasite viability, the following procedure was used to quantify dead and living parasites via direct microscopic observation.

The unstained parasites, living or dead, were injected according to the procedures described under Experimental. At the detector outlet, systematic fraction collections were performed, and every fraction was then analysed microscopically after concentration. Fig. 2 shows the elution pattern observed. The dead parasites are clearly more retained than the living parasites. This suggests that the motility of the living parasites will partially counteract the field effects. The parasite average diameter ranged from 14 to 18 μm, with an average value of 16 μm [12]. Moreover, its morphological types are round (35%) and ovoid (65%), and the average elution position of the living *Trichomonas* population in the channel thickness was less than twice this value ($R = 0.606$). This suggested that the parasites were eluted close to the accumulation wall. Not surprisingly, non-motile parasites are more highly retained, closer to the accumulation wall, with a retention factor of 0.309. According to the steric theory [1], the average position of the non-motile population centre of gravity was calculated to be 9 μm from the accumulation wall, and considering an average radius of 8 μm of the parasite, one can conclude that the flagellae motion of the living parasites moved them off the accumulation wall, driving the centre of gravity of the motile population up to 17.5 μm from the wall. Moreover, it appeared that living and dead parasites can be relatively well separated using GFFF, uniquely by the mean of the flagellae motion.

Previous work on motile and non-motile living species was described by Giddings [1], Jiang [13] and Jiang and Giddings [14], using sedimentation FFF (SdFFF) and *Escherichia coli*, and a close analogy with the data in this work can be observed. In both instances non-motile species are more retained, indicating that flagellae motility played a key role in the elution process. This analogy was found for two different methods of FFF, and for very different species, which eluted according to different elution modes (nor-

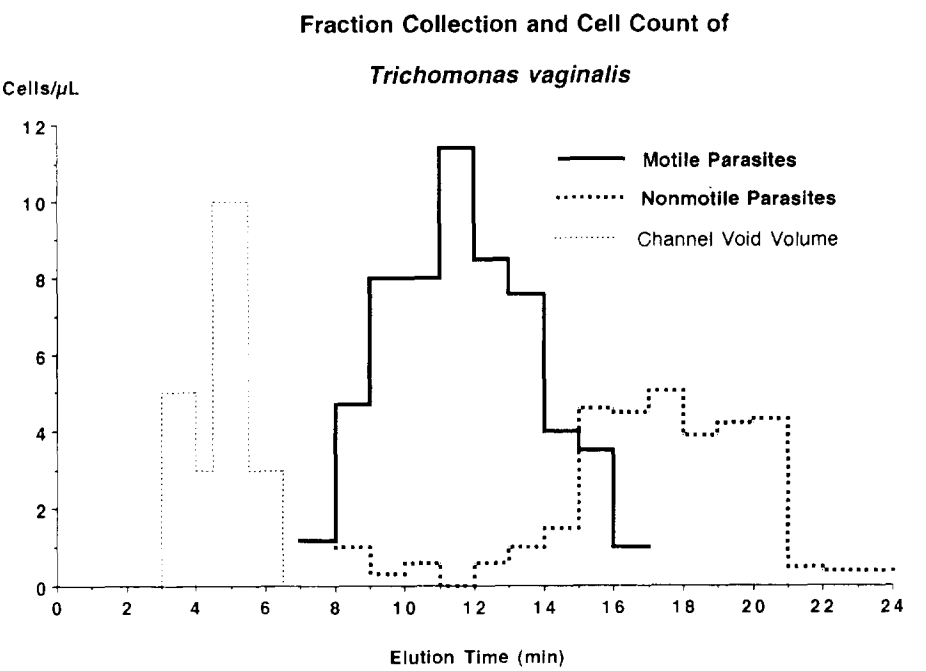

Fig. 2. Elution pattern of collected parasites after FFF elution. Eluted fractions are plotted for motile and non-motile parasites. The thin dotted line indicates the void volume elution pattern with arbitrary intensity, the solid line describes the motile parasite elution pattern and the thick dotted line describes the elution band of non-motile parasites. In each instance *Trichomonas vaginalis* was injected into the flow without staining. For each fraction, the collection time was 1 min and each fraction volume was reduced to 50 μl by centrifugation. Counting results are expressed in parasite number per microlitre of eluted carrier phase.

mal mode for *Escherichia coli* and steric mode for *Trichomonas vaginalis*).

3.3. FFF elution process and recovery

The first restriction of the methodological approach described here lies in the relatively low recovery of the dead *Trichomonas*, which limits the quantitative analysis of non-motile parasites. This problem could be solved by using a set of different accumulation wall materials; towards this end, empirical rules for biological materials are becoming known [2,4,5,10]. As is usually the case, the proportion of living parasites is the key factor required for pharmacological tests, but an estimate of the dead parasites will, in fact, be made. Methodologically, systematic sample concentration determination prior to injection has to be performed. The second restriction lies in the detection procedure, as cell staining is needed for photometric detection. The use of detectors more specifically designed for particles [15] can be employed and, in the case of cells, flow cytometric techniques will considerably enhance the detection criteria.

Finally, the undesirably long elution time observed in GFFF can be reduced using two approaches. The first approach tends to reduce the channel dimensions, as described by Yue et al. [16], and the second uses a stronger transverse field, such as by using SdFFF devices [8]. Such a procedure was first described for biological applications by Caldwell et al. [8]. Overall, the described approaches and results introduce new perspectives into analytical methods in the fields of parasitology and pharmacology, as predicted by Giddings [1]. With GFFF and *Trichomonas* it could be easy to study drug screening against *T. vaginalis* in culture broth or to detect metronidazole resistance of isolated strains cultured in metronidazole-supplemented broth. In particular, results from drug screening

in liquid medium could be simplified and, after recovery optimization, quantitative analysis of both motile and non-motile populations should be possible. Automated analysis of eluted dead and living parasite fractions using GFFF will be performed via on-line specific particle detectors. The enhancement of the threshold of sensitivity of the detection procedure would allow parasite detection in parasitized urines.

4. Conclusion

The preliminary results described in this paper suggest new biological applications of GFFF, not only as a diagnostic tool or as a specific device to study cell properties, but also as a pharmacological device as suggested in other parasite studies [3–4]. Previous experiments using SdFFF with motile and non-motile species such as *Escherichia coli* have been performed [1,13,14], this bacteria appeared to be at least ten times smaller than and with a shape completely different to that of *Trichomonas vaginalis*. The experiments performed in this work confirm the capability of FFF techniques to study biophysical features of biological materials. The GFFF design presented allows non-specialized laboratories to use FFF by simply replacing the column of an HPLC system by the GFFF channel. The possibility of sharing HPLC devices with GFFF can facilitate the introduction of such a separation system at relatively low cost or instrumentation skills. The methodology described here for *Trichomonas vaginalis* could be used as a screening system for new drugs allowing the 50%-efficiency concentration (E.C.50) determination. Moreover, this methodology can be extended to all types of motile uni- or multicellular species.

Optimization rules for channel geometry in FFF have not been established. All the reported approaches to limiting particle–wall interactions have been empirical and it is difficult to assess this problem. More extended experimental studies should be performed in terms of instrumentation, as the most critical part of FFF elution lies in the non-negligible particle–wall interactions obtained with different types of cellular material, which partially limits quantitative analysis. For example, polycarbonate plates could be compared with siliconized glass plates, the latter surface appearing to be much more hydrophobic.

Acknowledgements

Our sincere thanks are due to Dr. R. Parsons of Abbot Laboratories (Chicago, IL, USA), who provided us with buffer materials and the carrier phase systematically used in this work, and also to B. Paulet and Mrs. J. Robic for technical assistance. Luigi P. Reschiglian of the Analytical Chemistry Department, University of Bologna (Bologna, Italy) is acknowledged for reading the manuscript and for critical comments.

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