Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1056 (2004) 229–236
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Field-flow fractionation of cells with chemiluminescence detection

Dora Melucci[a,*], Barbara Roda[a], Andrea Zattoni[a], Sonia Casolari[a], Pierluigi Reschiglian[a], Aldo Roda[b]

[a] *Department of Chemistry "G. Ciamician", University of Bologna, Via Selmi 2, I-40126 Bologna, Italy*
[b] *Department of Pharmaceutical Sciences, University of Bologna, Via Belmeloro 6, I-40126 Bologna, Italy*

Available online 15 September 2004

Abstract

Field-flow fractionation is a separation technique characterized by a retention mechanism which makes it suitable for sorting cells over a short analysis time, with low sample carry-over and preserving cell viability. Thanks to its high sensitivity, chemiluminescence detection is suitable for the quantification of just a few cells expressing chemiluminescence or bioluminescence. In this work, different formats for coupling gravitational field-flow fractionation and chemiluminescence detection are explored to achieve ultra-sensitive cell detection in the framework of cell sorting. The study is carried out using human red blood cells as model sample. The best performance is obtained with the on-line coupling format, performed in post-column flow-injection mode. Red cells are isolated from diluted whole human blood in just a few minutes and detected using the liquid phase chemiluminescent reaction of luminol catalysed by the red blood cell heme. The limit of detection is a few hundred injected cells. This is lower than the limit of detection usually achieved by means of conventional colorimetric/turbidimetric methods, and it corresponds to a red blood cell concentration in the injected sample of five orders of magnitude lower than in whole blood.

Keywords: Field-flow fractionation; Chemiluminescence detection; Flow-injection analysis; Human red blood cells

1. Introduction

Analysis of complex biological matrices usually requires to clean up the sample or to partially isolate the analyte before instrumental analysis. In the case of molecules in complex matrices, several well-established separation techniques such as HPLC or capillary electrophoresis are available. On the contrary, in the case of cells or, in general, particulate matter present in complex mixtures such as biological fluids, the availability of selective and rapid separation techniques is still requested. Preservation of cell morphology during separation is also desirable.

As far as living cell characterization is concerned, rapid, specific, sensitive cell sorting methods are needed for many purposes, including the identification of hazardous micro-organisms for zoo-prophylaxis and counteracting bio-terrorism, isolation of bacteria and viruses for vaccine production, isolation of disease-marker cells for diagnostics and viable cells for therapeutics. Many systems have been developed for sorting, counting and sizing cells. Flow cytometry is a standard technique in biology. Signal from the forward angle as well as side scattering allow for cell characterization [1,2]. Techniques based on microelectrode arrays combining electrokinetic and hydrodynamic forces are also available for cell isolation and cell sizing. All of these techniques present many drawbacks including the need for expensive, complex instrumentation and, in particular for flow cytometry, also the need for large sample volumes with the inherent risk of contamination and sample waste [3,4]. Other techniques for cell analysis involve elutriation and density gradient sedimentation procedures to separate cell subpopulations, or the immunomagnetic separation procedure based on the use of antibodies immobilized on magnetic particles. The latter technique, for example, is applied to the selection of lung fibroblast populations [5,6]. With respect to cell sorting, these procedures may alter cell natural morphology and require a labor-intensive sample preparation; all of which are, indeed, significant drawbacks.

* Corresponding author. Fax: +39 051 2099 456.
E-mail address: dora.melucci@unibo.it (D. Melucci).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.08.084

Field-flow fractionation (FFF) is a family of flow-assisted separation techniques based on the combined action of a transporting laminar flow within an empty capillary channel and of a field applied perpendicularly to the flow. The field can discriminate particulate samples having similar physico-chemical properties but different size and morphology. Thanks to this peculiar separation mechanism, short analysis time and the ability to perform separation under sterile conditions, FFF has already been used to take advantage in cell sorting [7–14]. Disposable, micro-column FFF channels for cell fractionation have recently been described [14,15]. One of the major weakness of FFF methods for cell characterization has, however, been cell detection methods, which in some cases should be extremely sensitive to detect cells with some specific features among a low amount of cells loaded into the FFF channel. In FFF, detection is commonly based on the use of a turbidimetric or light scattering detector. In both cases, there is lack of detection specificity and sensitivity due to the aspecific, complex interaction that takes place between the incident light and the heterogeneous sample/dispersing medium system.

In chemiluminescence (CL) detection, the light can be specifically generated by the reacting molecules, and the resulting cold light can be measured with highly sensitive instrumentation such as CCD cameras or photomultiplier tubes (PMT) devices able to detect even just a few emitted photons. This means that, thanks to the overall quantum efficiency of a given CL reaction, they can detect the few reacting molecules present in the sample. The CL signal is generated in the dark, there is no incident light, and scattering effects in the analytical cell do not affect measurements. Moreover, the analytical signal is selectively triggered by a specific catalyst, thus avoiding aspecific light as in photoluminescence spectroscopy. Moreover, it has been previously demonstrated that liquid phase CL detection is suitable for hyphenation with many separation techniques including HPLC, capillary electrophoresis and flow-injection analysis (FIA) [16,17], and many bio-analytical applications have been reported [18].

Cells can exhibit spontaneous bioluminescence (BL) (e.g. bioluminescence of some marine bacteria), or BL as a consequence of manipulation such as surface labeling or genetic modification [18–20]. In addition, cell components can be coupled with a proper substrate in order to generate CL. For instance, ATP gives CL with the luciferin/luciferase system; endogenous intracellular alkaline fosfatase is an enzyme which catalyses the CL hydrolysis of dioxiethane phosphate substrates. In the present work, human red blood cells (HRBCs) are used as model-samples to investigate the feasibility of FFF-CL for specific, sensitive cell sorting. The morphology and composition of HRBCs are well characterized. They can be sorted by FFF as demonstrated by Cardot and coworkers [9,21] and Tong and Caldwell [22] and the dependence of their morphology on the dispersing medium composition (in particular pH and ionic strength) is known. HRBCs contain hemoglobin whose prosthetic group – the heme-ferrous complex – is a catalyst for the luminol-peroxide CL reaction extensively used in forensic analysis [23,24]. The CL reactions of luminol-type reagents are also extensively used for bio-analytical techniques since they present many advantages over conventional spectrophotometric techniques: faster kinetics and higher sensitivity. Moreover, it generates a steady-state CL signal which remains constant for several minutes [25], e.g. long enough to perform FFF sorting [26].

In previous studies [26,27], we demonstrated that micrometer-sized polystyrene (PS) spheres bound to horseradish peroxidase (HRP) can be analyzed by FFF-CL, either off-line or in continuous mode. In the first case the CL cocktail (luminol/H_2O_2/*p*-iodophenol) was added after particle sorting into different collected fractions; then the CL signal was measured using a microtiter plate luminolmeter [27]. In the second case, the CL cocktail was added directly to the mobile phase and the CL signal continuously recorded using a flow-cell luminolmeter with a flow-through cell. The micrometer-sized PS-HRP particles can simulate cells containing a CL catalyst: the mean diameter of HRBCs is 6.6 μm and they contain the heme-ferrous catalyst for the luminol-peroxide CL reaction.

The present work determines the best instrumental set-up and the best composition of the solution (the so-called CL "cocktail") to generate the CL signal from HRBCs to make HRBCs be effectively detected in FFF-CL at low limit of detection (LoD). In particular, different modes of coupling CL detection with FFF separation have been investigated. Off-line, on-line in continuous mode or on-line in post-column flow-injection mode are the FFF-CL configurations explored. Gravitational FFF (GrFFF) has been selected because it is simple to use, it employs low-cost instrumentation, and its performance and high sample throughput for HRBC fractionation is well established [28–30].

2. Experimental and methods

2.1. GrFFF

The fractionator was home-built as previously reported [13]. The channel design was specifically developed for the fractionation of samples of biological origin where sterility and recovery are critical parameters. The depletion wall was made of polycarbonate (PC), the accumulation wall of polyvinyl chloride (PVC) characterized by high biocompatibility and low cell-wall interaction, as demonstrated by Cardot and coworkers [21]. Channel thickness, length and breadth were, respectively, 0.0180 cm × 30 cm × 2.0 cm. The nominal channel surface and volume were, respectively, 55 cm^2 and 0.99 cm^3. The carrier flow was delivered by a Varian Model 2510 pump (Varian, Walnut Creek, CA). Samples were injected into the GrFFF channel by a Rheodyne Model 7125 valve (Rheodyne, Cotati, CA) equipped with a 5 μL PEEK loop. Samples were injected for 20 s at a flow rate of 0.2 ml min^{-1} and then the flow was stopped for 3 min

to allow for sample relaxation (stop-flow). At the end of the stop-flow time, sample elution was started at a flow rate of 0.6 ml min^{-1}.

2.2. Detection

Two different UV–vis detectors were employed as turbidimeters: the UV 6000 LP (ThermoQuest, Austin, TX), a high-sensitivity diode-array UV–vis detector equipped with a fiber optic guide light-pipe cell whose path length was 5.0 cm, and the UV–vis detector Dynamax Model UV-1 (Varian) operating at 600 nm and equipped with a standard 1 cm long optical cell.

The CL signal was measured by an FB12 luminolmeter (Berthold Detection Systems, GmbH, Pforzheim, Germany). Operating in the 370–630 nm spectral range, this luminolmeter adopts a photomultiplier tube technology characterized by high stability and low-noise electronics. It was connected to a PC with a serial RS-232 interface, and the CL signal was acquired using the proprietary data acquisition software FB12 Sirius Software (Berthold Detection System), with 0.2 s sampling time. Results are presented in relative light units (RLU) s^{-1}. The FB12 luminolmeter was originally designed for static CL measurements, using standard 12 mm × 75 mm tubes. For on-line GrFFF-CL measurements, the sample vial was home-modified to include a coiled 1 mm i.d. transparent teflon tube. This made it possible to assemble an original 50 μL cell for flow-through CL detection. The vial containing the coil was set in the sample drawer of the luminolmeter. The flow-through CL detector inlet was connected to the outlet of the UV–vis detector.

2.3. GrFFF-CL modes

Three modes were developed for coupling the FFF channel and the CL detector: off-line GrFFF-CL, on-line continuous GrFFF-CL and on-line post-column flow-injection GrFFF-CL.

In the off-line GrFFF-CL mode, 60 μL fractions of the GrFFF effluent were collected from the outlet of the UV–vis detector in 2 ml plastic vials (Eppendorf AG, Hamburg, Germany). Six microliters of the CL cocktail were added to each fraction. Each vial, containing both the sample fraction and the CL cocktail, was stirred for 20 s and then placed into the sample holder for static CL signal measurements.

The continuous GrFFF-CL mode was an on-line mode: the mobile phase contained the CL cocktail to generate the CL reaction while samples were eluting.

A GrFFF-FIA-CL coupling was first suggested by other authors for the determination of metals adsorbed on particulate matter [31]. In the GrFFF-FIA-CL configuration specifically developed for this study, the CL cocktail was post-column flow-injected by means of a low swept volume (2.2 μL) "tee" reactor, located downstream of the UV–vis detector and upstream of the flow-through luminolmeter. The CL solution was delivered by means of a Miniplus 3 Model peristaltic pump (Gilson, Middleton, WI) at a flow rate of 0.06 ml min^{-1} (10% of mobile-phase flow rate).

2.4. Chemicals

Tris(hydoxymethyl)amminomethane (Tris), sodium chloride, sodium dodecyl sulfate (SDS), *p*-iodophenol, hydrogen peroxide, sodium carbonate, sodium borate, sodium cholate (sodium 3α, 7α12α-trihydroxy-5β-cholan-24-oate), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione sodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO). ECL® was from Amersham Pharmacia Biotech, UK.

2.5. Samples and mobile phases

Fresh human blood samples were drawn from a healthy donor and K_3EDTA was added as anticoagulant. Blood was injected as such or pre-treated. In both cases, whole blood was diluted in a physiological Tris-buffered solution (TBS, Tris–Cl 10 mM, NaCl 140 mM, pH 7.4) prior to injection. The pre-treatment, repeated three times, consisted of: washing, centrifuging and re-suspending the HRBCs in physiological TBS to remove blood plasma and free hemoglobin. The samples were stored at 4 °C. The injected HRBCs were in the range of 100–10,000 cells per run. The HRBC concentration for all the blood samples was obtained by standard methods of clinical analysis.

The mobile phase was physiological TBS or TBS at pH 8.6 which also contained 1 mM sodium cholate. We have previously shown that, when present in the mobile phase, cholate is able to minimize cell-wall interactions and to improve recovery of HRBCs fractionated by hollow-fiber flow field-flow fractionation [15]. The sodium cholate concentration here employed was far below its critical micellar concentration (CMC: 11 mM): under this condition HRBC lysis during elution is in fact negligible [32].

Whole or lysed HRBCs were employed for CL measurements. Cell lysis was obtained by osmotic shock, either by dispersing the cells in a diluted solution of NaCl (0.3% (w/v)) or by adding SDS to the dispersing medium at a concentration (3% (w/v)) far above the critical micellar concentration (CMC: 0.3% (w/v)).

2.6. Chemiluminescent systems

Three different CL "cocktails" were used (see Table 1). *Cocktail 1* was prepared in the laboratory. The composition of this cocktail was optimised in a previous work for the detection of PS microspheres bound to HRP [26]. It was prepared by adding 1 mM of luminol to the mobile phase and using 10 μM *p*-iodophenol as enhancer. Then, 1 mM hydrogen peroxide was added to the solution just a few minutes before the analysis. *Cocktail 2* was ECL®, a commercial luminol-based substrate, used here as a reference standard for HRBC detection by CL. *Cocktail 3* was prepared by modifying a CL cocktail described in the forensic chemistry literature for

Table 1
Calibration plot, sensitivity and LoD for the CL signal from HRBCs

Cell pretreatment	CL cocktail	Intercept (RLU s^{-1})	Sensitivity (RLU s^{-1} $cell^{-1}$)	Correlation coefficient	LoD (cells/tube)
Washing	Cocktail 1: luminol/H_2O_2/*p*-iodophenol	$8 \times 10^4 \pm 2 \times 10^5$	$1.00 \times 10^3 \pm 9 \times 10^1$	0.9962	200
Washing and lysis	Cocktail 1: luminol/H_2O_2/*p*-iodophenol	$4 \times 10^5 \pm 9 \times 10^5$	$1.3 \times 10^4 \pm 2 \times 10^3$	0.9796	100
Washing and lysis	Cocktail 2: ECL	$5 \times 10^5 \pm 1 \times 10^6$	$3.1 \times 10^4 \pm 4 \times 10^3$	0.9835	80
Washing and lysis	Cocktail 3: luminol/$NaBO_3$/Na_2CO_3	$1 \times 10^5 \pm 3 \times 10^5$	$1.25 \times 10^4 \pm 8 \times 10^2$	0.9949	70

the ultra-trace determination of blood in crime scenes [23,24]. This cocktail contained luminol 5 mM, Na_2CO_3 430 mM and $NaBO_3 \cdot 4H_2O$ 46 mM.

3. Quantitative and statistical analysis

3.1. CL kinetics

In the case of static measurements, all the CL *cocktails* tested exhibited similar kinetics: after adding the CL substrate, a rapid increase in signal was observed, then a steady state was rapidly reached and maintained for longer than the measurement time. The time interval required to reach the steady state increased as the concentration decreased (Fig. 1). Nonetheless, for all concentrations, the steady-state signal was reached in less than 0.2 s, which was the selected delay time for the luminolmeter data acquisition. The steady-state intensity differed among the three CL *cocktails* tested; maximum intensity was achieved using the luminol/borate system (*Cocktail 3*). In all cases, CL signal intensity depended only on the amount of heme, the hydrogen peroxide being in excess.

In the case of GrFFF-FIA-CL measurements, the analytes eluted from the FFF channel and mixed with a CL *cocktail* containing solution reached the luminolmeter flow-through cell when the CL signal was at the steady-state level, which was maintained longer than the residence time.

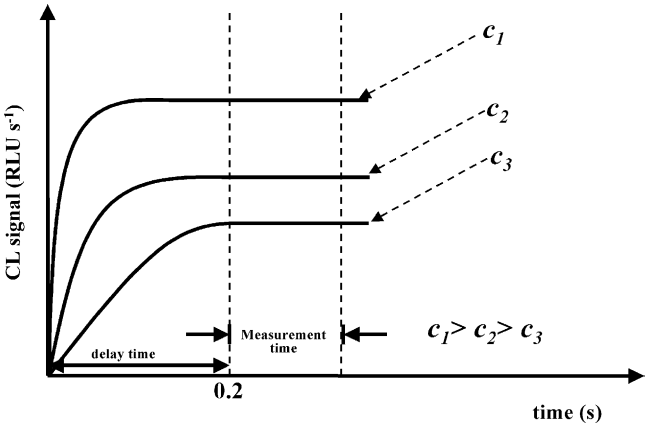

Fig. 1. CL signal kinetics as a function of the luminescent-analyte concentration *c*.

3.2. Quantitative analysis by flow-through CL measurements

In the hypothesis that the CL signal is linear with the number of cells, we can write:

$$I = Kn \tag{1}$$

where I (RLU s^{-1}) is the intensity of the CL-emitted light, K (RLU s^{-1} $cell^{-1}$) a proportionality constant and n (cell) is the number of cells in the assay tube. This hypothesis can be checked by applying a linearity test to I versus n data.

In the case of on-line, flow-through CL measurements, n and, therefore, I are time dependent. It has been demonstrated [33,34] that when a time-dependent signal produced in a flow-through analytical cell is proportional to the time-dependent quantity of the analyte passing through that cell, the integrated signal is proportional – with the same proportionality coefficient – to the total amount of analyte that has passed through the analytical cell multiplied by its average residence time within the cell. The latter is, in turn, equal to the ratio between the cell volume and the flow-rate. As a consequence, in the case of the CL signal subject to Eq. (1), under a steady-state stable analyte-dependent CL signal, we can write:

$$\hat{I}F = KV_{\mathrm{LUM}}n_0 \tag{2}$$

where $\hat{I}$ (RLU) is the fractographic peak area, F (ml s^{-1}) the mobile-phase flow-rate, V_{LUM} (ml) the luminolmeter cell volume and n_0 (cell) is the number of eluted cells. Eq. (2) is obtained from Eq. (1) in the same way as the Beer-Lambert-like law for heterogeneous flow-through systems is obtained from the Beer-Lambert law [33]. In Eq. (2), $\hat{I}$ can be replaced by the product obtained by multiplying peak height (I_{MAX}) by peak width at half height ($w_{1/2}$), and I_{MAX} can be written (Eq. (1)) as Kn_{MAX}, where n_{MAX} is the number of cells simultaneously present in the luminolmeter flow-cell at the peak maximum. The following equation for the evaluation of n_{MAX} is, thus, obtained:

$$n_{\mathrm{MAX}} = \frac{V_{\mathrm{LUM}}}{w_{1/2}F}n \tag{3}$$

Accurate quantitative evaluation requires a well-standardized, constant flow rate. Actually, a precision better than 1% and a trueness at a 5% level of significance were always verified in flow-rate calibration.

3.3. Recovery and limit of detection

The estimation of the absolute recovery of fractionated cells is of fundamental importance in the development of all FFF-based cell sorting methods. For all our GrFFF-CL modes, the absolute recovery of HRBCs was evaluated as the ratio between the eluted peak area and the peak area obtained for the same amount of sample directly injected into the UV–vis detector cell without fractionation in the GrFFF channel [35,36]. In all cases, the average absolute recovery was 80%.

The limit of detection for batch measurements was evaluated from a linear calibration plot (signal versus quantity), as the ratio between $3s_{y/x}$ and the slope. In the case of GrFFF measurements, because of the non total absolute recovery, the LoD was evaluated from the signal-to-noise ratio of the fractograms, as described in a previous work [26]. For all statistic calculations, a 95% confidence level was chosen.

3.4. Statistical test for linearity

When the linear dependence of the analytical signal on analyte quantity has no physical foundation, linearity can be checked by a statistical procedure. A well established test for linearity is the ANOVA test which compares the variability within groups of replicate measurements (pure experimental uncertainty, or pure error (PE)) and the variability due to the lack of fit (LoF) [37]. The F_A parameter was calculated as the ratio of the LoF variance to the PE variance. When the experimental value of F_A exceeds the critical, tabulated value, the hypothesis of linearity is rejected, otherwise linearity is assessed. For all statistic calculations, a 95% confidence level was chosen.

4. Results and discussion

4.1. On-line CL linearity

In order to check the linearity range for the CL signal, solutions of HRP in the mobile phase at different concentrations were directly injected into the flow-through luminolmeter through the six-way injection valve. The mobile phase chosen for this study was *Cocktail 1*, the same cocktail used in the previous FFF-CL work on PS-HRP [26]. Five HRP standards (from 1 to 5 in increasing concentration order) were injected, and each injection was repeated six times. The injected mass was between 0.2 and 3 ng. An ANOVA test was performed to check for lack of fit [37] versus linear function. Weighted linear regression was performed. Lack of fit is highlighted when all five experimental points are considered: the calculated F_A value was 30.2, which exceeds the critical value, equal to 3.01. On the contrary, the hypothesis of linearity holds true if the highest injected-mass experimental point is removed: calculated F_A results to be equal to 2.49, which is lower than the tabulated value, equal to 3.55. This means that points 1–4 fall within the linear range. The highest observed peak height value inside the linear range was 2.4×10^7 RLU s^{-1}. It must be pointed out that the CL signal relevant to the linear range covers seven orders of magnitude, while the turbidimetric signal in the same range covers only one order of magnitude.

4.2. CL of HRBC: sensitivity and limit of detection

To check sensitivity and limit of detection for the CL signal obtained from cells, static CL measurements were performed. Various HRBC dispersions were prepared in different CL cocktail concentrations, and static CL measurements were repeated three times on each dispersion. The static CL signal values were within the linear range for the PMT luminolmeter. A calibration plot was calculated through a linear regression of the CL signal (RLU s^{-1}) versus the number of dispersed cells. All the thus-obtained static measurements are listed in Table 1. In all cases, the correlation was good and the intercept not significantly different from zero.

4.2.1. Effect of cell lysis

Cocktail 1 was used in two experiments, the first with whole HRBCs, the second with lysed cells. Table 1 shows that cell lysis improves the CL detection sensitivity. The relevant LoD was also improved. However, in the case of lysed cells, the reduction in LoD was not proportional to the increase in sensitivity. This was due to the fact that the correlation coefficient obtained in lysed cells was lower than in the case of whole cells. This gave a higher value of $s_{y/x}$ and, consequently, a LoD value worse than the one expected for the increase in sensitivity achieved.

4.2.2. Effect of CL cocktail composition

To evaluate its analytical performance, *Cocktail 1* was compared with a commercial, high performing CL system (*Cocktail 2*). Data obtained with lysed HRBCs were compared. With *Cocktail 2* sensitivity is indeed higher, and LoD lower. Nevertheless, one of the main drawbacks in the possible use of *Cocktail 2* is that its chemical composition is patented and, thus, unknown. This makes it difficult to optimise the use of *Cocktail 2* for the various GrFFF-CL configurations explored in this paper. For instance, this information is often needed for a precise control of either the pH or excesses in the real CL substrate versus the number of cells. Since *Cocktail 2*, however, performed better than *Cocktail 1*, a third cocktail of known composition and with performance similar to *Cocktail 2* was sought. *Cocktail 3*'s performance was good: sensitivity and LoD were comparable to those obtained with *Cocktail 2*.

The results described in Table 1 provide full information about the minimum number of cells simultaneously present in the flow-through luminolmeter cell that can be detected in the various combinations of cell pre-treatment and CL-cocktail composition.

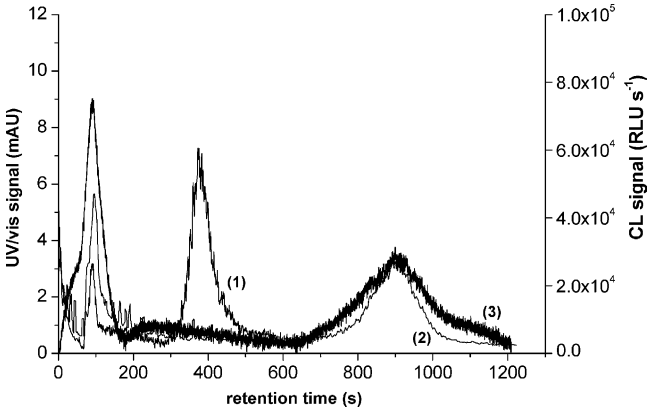

Fig. 2. Continuous GrFFF-CL. Mobile phase: TBS, sodium cholate 1 mM, luminol 1 mM, *p*-iodophenol 10 μM, H_2O_2 1 mM. Sample: diluted natural blood; 10,000 injected HRBCs. (1) UV/vis DAD signal, λ = 600 nm, pH 7.4; (2) UV–vis DAD signal, λ = 600 nm, pH 8.6 and (3) CL signal, pH 8.6.

4.3. GrFFF-CL of HRBCs

4.3.1. Continuous GrFFF-CL mode

In a previous work [26], micrometer-sized PS spheres bound to HRP were successfully fractionated in GrFFF and detected by CL in continuous mode, that is using a mobile phase containing the CL cocktail for the CL reaction catalysed by HRP. The cocktail used therein was *Cocktail 1* and the mobile-phase had a pH of 8.6.

In the case of HRBC, however, application of the continuous mode is not straightforward. In fact, the CL reaction between luminol and a peroxide, catalysed by hemoglobin, requires alkaline conditions. In principle, it is possible to elute HRBCs with an alkaline mobile phase, but this would alter cell morphology [32]. When dispersed in alkaline liquids, the HRBC morphology changes from a disc-like shape (the erythrocytes) to a sphere-like shape with external protrusions (the echinocytes). Moreover, modification of cell properties such as cell-membrane rigidity and cytoplasm composition cannot be ruled out. These modifications can induce a variation in HRBC retention, as confirmed by the experiments reported in Fig. 2. The GrFFF-UV–vis fractogram obtained with the physiologic mobile phase (case 1) is completely different from the GrFFF-UV–vis fractogram obtained when the pH of the same mobile phase is raised to 8.6 (case 2). Moreover, the fractogram obtained in continuous GrFFF-CL mode (case 3) shows no gain in the signal-to-noise ratio with respect to the GrFFF-UV/vis fractograms (cases 1 and 2).

The results shown in Fig. 2 suggest that the continuous mode should be avoided and that a post-GrFFF addition of the CL cocktail prior to CL detection be tested instead. Moreover, the results reported in Table 1 also provide clear indication that lysis of the HRBCs prior to addition of the CL cocktail can improve the CL signal. Once the possibility of working in continuous GrFFF-CL mode was ruled out, the post-column, flow-injection mode (GrFFF-FIA-CL) was recognized as the only real possibility for on-line GrFFF-CL of HRBCs.

4.3.2. Off-line GrFFF-CL

Off-line GrFFF-CL was preliminarily considered in order to achieve the best conditions for the post-column addition of the CL cocktail and for the composition of the lysing agent. However, this configuration presents several drawbacks due to the possible loss of separation caused by remixing of the eluted analytes in the collected fractions. In addition, this approach is time-consuming. On the other hand, in off-line mode HRBCs were eluted in a physiological mobile phase and the CL/lysis cocktail was added to the different fractions collected in separate vials. In order to achieve the best optimisation conditions, the following parameters were adjusted: CL cocktail composition, ratio between the volume of the GrFFF collected fractions (V_F) and the volume of the added CL cocktail (V_A), lysis-agent composition. The optimum V_F/V_A ratio indicates the optimum proportion between the volume per unit of time for the mobile phase exiting the GrFFF channel and the volume per unit of time for the liquid added to the mobile phase in post-column mode. In other words, the optimum V_F/V_A ratio is equal to the optimum ratio between the elution flow rate and the flow rate of the CL/lysis cocktail to be added in post-column FIA. Because of the high osmolarity of the physiological mobile phase, in GrFFF-FIA-CL cell lysis could not be carried out by osmotic shock. Carrying out osmotic shock in post-column FIA mode would have required a very high FIA flow rate. For this reason, lysis was performed by adding a surfactant to the CL cocktail.

After several trials, the following experimental conditions for off-line GrFFF-CL were selected

(1) The best CL cocktail was obtained from *Cocktail 3* by a five-fold increase in the luminol and perborate concentrations.
(2) The V_F/V_A ratio was set equal to 10.
(3) Lysis was performed by adding to the CL cocktail SDS at a concentration equal to 3% (w/v) which is above its CMC.

The above conditions gave the results reported in Fig. 3. It is shown that, for the same number of injected cells, the CL signal is far above the minimum detectable signal (see Table 1), while the UV–vis signal is just a few mAU, close to the minimum detectable signal for standard UV–vis detectors used as turbidimeters. In fact, it must be pointed out that a high-sensitivity UV–vis detector was employed in the experiment reported in Fig. 3. This detector was equipped with a 5 cm, light-pipe cell that, in principle, gives detection sensitivity five times higher than the sensitivity of standard UV–vis detectors equipped with conventional 1 cm long Z-type cells. Therefore, a UV–vis signal for a number of injected cells as in Fig. 3 would have hardly been obtained with standard UV–vis detectors.

4.3.3. On-line GrFFF-FIA-CL

The best conditions found in the off-line experiments were used to determine the best experimental conditions in post-

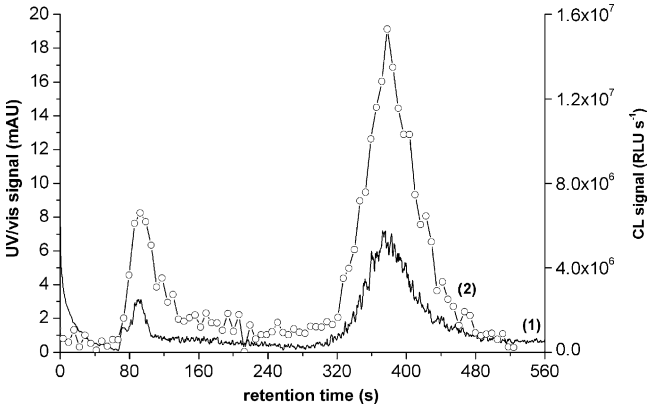

Fig. 3. Off-line GrFFF-CL. Mobile phase: physiological TBS, sodium cholate 1 mM. Sample: washed HRBCs, 7000 injected cells. CL/lysis cocktail: 25 mM luminol, 430 mM Na_2CO_3, 230 mM $NaBO_3$, SDS 3% (w/v). (1) UV/vis DAD signal, λ = 600 nm and (2) CL signal.

column FIA-CL. The CL/lysis cocktail was prepared from *Cocktail 3*. The luminol and perborate concentration was not increased five-fold as in the off-line experiments because, when flow-injected, the concentrated *cocktail* was not stable enough for the longer duration that a GrFFF-FIA-CL experiment requires over an off-line experiment.

The CL/lysis *cocktail* was flow-injected through a peristaltic pump. Results are reported in Fig. 4. It is shown that when the number of injected HRBCs was approximately 500 – a 50,000-fold dilution of the whole blood sample – the CL signal is still appreciable while the turbidimetric signal is not significantly different from the baseline noise. Hence, the CL detection here applied to HRBCs is not only specific for these cells, but also more sensitive than the non specific UV–vis turbidimetric detection. The LoD for GrFFF-FIA-CL was equal to 300 injected HRBCs. Using Eq. (3) it is possible to estimate that a few hundred injected cells in fact correspond to about 10 cells at the peak maximum, which is a very high detection sensitivity in the case of GrFFF of HRBCs.

4.3.4. GrFFF-CL versus GrFFF-UV–vis sensitivity

The sensitivity of the GrFFF-FIA-CL system and of the GrFFF-UV–vis system was evaluated using the ratio of the difference between the detector response obtained with two different numbers of injected cells and the difference between the number of injected cells. It resulted to be approximately equal to 1000 RLU $cell^{-1}$ and 0.02 mAU $cell^{-1}$, respectively. These values demonstrate that, in the case of cells, CL detection can effectively enhance sensitivity over that obtained with UV–vis turbidimetry. Moreover, the analytical signal is generated without a light source through a specific chemical reaction. UV–vis turbidimetric detector indeed measures light scattering as a function of cell size and morphology, while the CL detector measures light coming from cells working as though they were themselves light sources, as a consequence of the CL reaction of luminol catalysed by the red cell heme. It should be pointed out that a calculation of the

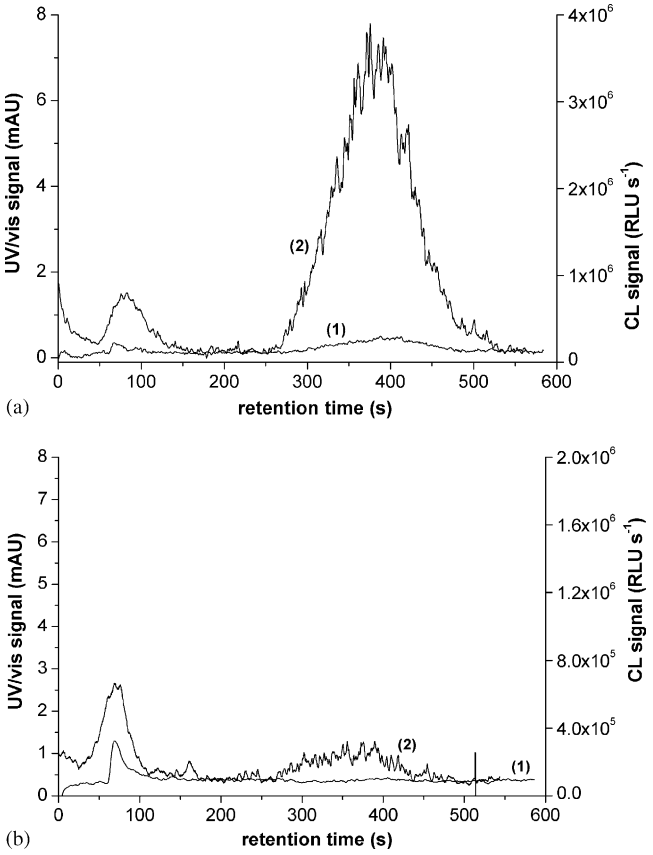

Fig. 4. GrFFF-FIA-CL. Mobile phase: physiological TBS, sodium cholate 1 mM. Sample: washed HRBCs (a) 5000 injected cells and (b) 500 injected cells. CL/lysis cocktail: luminol 5 mM, Na_2CO_3 430 mM, $NaBO_3$ 46 mM, SDS 3% (w/v). (1) UV/vis signal, λ = 600 nm and (2) CL signal.

amount of hemoglobin present in a single cell should make it possible to determine the efficiency of the system photon emission, thus giving the number of molecules actually responsible for the CL signal measured in a GrFFF-FIA-CL system. This evaluation would give an idea of the cell signal amplification and sensitivity but goes beyond the scope of this paper.

5. Conclusions and perspectives

This work is a feasibility study with a model sample of cells. It aims at demonstrating that, under experimental conditions which do not cause any alteration of cell morphology during separation, GrFFF-CL can perform cell fractionation at very low limits of detection. The GrFFF-FIA-CL mode eventually gave very satisfying results, detecting as low as a few hundred HRBCs contained in human blood. This corresponds to the detection limit of cells obtained after a 5×10^4-fold dilution.

In perspective, this study will be applied to the general problem of isolating a few specific, viable cells from complex natural matrices of different origin. Some cells express natural bioluminescence activity. A general way to make BL

from cells is also to bind a proper CL tracer to the cell membrane. Cells can also express BL when transfected by a gene which codes for the synthesis of a CL-reaction enzyme; an example of this is luciferase whose synthesis is driven by a promoter gene normally activated in response to specific or general environmental changes. These "biosensors" are used for many important applications such as drug screening, environmental and food-industry applications. We shall investigate all these cases within the framework of low-LoD cell sorting approaches using FFF-CL. Such a goal could have important applications in clinical treatment and diagnosis (e.g. transplant of stem cells, early identification of cancer-markers). Optimisation to seek the highest sample recovery conditions shall increase capabilities to detect a lower number of injected cells. Miniaturization of the FFF device is also possible and applying CL detection will even make it possible to measure and image the light coming from samples from the micrometer down to the nanometer size range (e.g. from cells to proteins) with very low amounts of injected sample.

Acknowledgments

The authors wish to thank Guido Nincheri for the experimental work and Marco Montagnani (Department of Internal Medicine and Gastroenterology, University of Bologna) for having kindly supplied the blood samples. Thanks also go to Massimo Guardigli (Department of Pharmaceutical Sciences, University of Bologna) for the helpful suggestions on CL signal handling, and to Eileen Nan Cartoon for English revision. Suggestions given by P. Cardot (University of Limoges) on biocompatible GrFFF channel set-up are duly acknowledged. Grants: This work was financially supported by the Italian Ministry of the Education, University and Research (MIUR, Ministero dell'Istruzione, dell'Università e della Ricerca, COFIN 2000 (MM03247343-005)).

References

[1] H.M. Salzman, S.B. Singham, R.G. Johnston, C.F. Bohren, in: M. Melamed, T. Lindmo, M.L. Mendelshon (Eds.), Flow Cytometry and Sorting, second ed., Wiley, New York, 1989, p. 81.
[2] A.L. Koch, B.R. Robertson, D.K. Button, J. Microbiol. Methods 27 (1996) 49.
[3] N. Borth, M. Zeida, R. Kunert, H. Katinger, Biotechnol. Prog. 135 (1999) 953.
[4] K.L. Chan, H. Morgan, E. Morgan, I.T. Cameron, M.R. Thomas, Biochim. Biophys. Acta 1500 (2000) 313.
[5] L.M. Wahl, I.M. Katona, R.L. Wilder, C.C. Winter, B. Haraoui, I. Scher, S.M. Wahl, Cell Immunol. 85 (1984) 373.
[6] E.W. de Winter, L.H. Coutinho, X. Pei, J.C.W. Marsh, J. Hows, T. Luft, N.G. Testa, Stem Cells 13 (1995) 524.
[7] K.D. Caldwell, Z.-Q. Cheng, P. Hradecky, J.C. Giddings, Cell Biophys. 6 (1984) 233.
[8] E. Urbankova, A. Vacek, J. Chmelík, J. Chromatogr. B 687 (1996) 449.
[9] A. Lucas, F. Lepage, Ph.J.P. Cardot, in: M.E. Schimpf, K.D. Caldwell, J.C. Giddings (Eds.), Field-Flow Fractionation Handbook, Wiley-Interscience, New York, NY, 2000 (Chapter 29).
[10] S. Rasouli, E. Assidjo, Ph.J.P. Cardot, J. Chromatogr. B 754 (2001) 11.
[11] S. Battu, A. Roux, S. Delebasee, C. Bosgiraud, Ph.J.P. Cardot, J. Chromatogr. B 751 (2001) 131.
[12] C. Lautrette, P.J.P. Cardot, C. Vermut-Descroches, J. Wijdenes, M.O. Jauberteau, S. Battu, J. Chromatogr. B 791 (2003) 149.
[13] P. Reschiglian, A. Zattoni, B. Roda, S. Casolari, M.H. Moon, J. Lee, J. Jung, K. Rodmalm, G. Cenacchi, Anal. Chem. 74 (2002) 4895.
[14] P. Reschiglian, B. Roda, A. Zattoni, B.-R. Min, M.H. Moon, J. Sep. Sci. 25 (2002) 490.
[15] P. Reschiglian, A. Zattoni, B. Roda, L. Cinque, D. Melucci, B.R. Min, M.H. Moon, J. Chromatogr. A 985 (2003) 519.
[16] N. Kuroda, M. Kai, K. Nakashima, in: A.M. Garcia-Campaña, W.R.G. Baeyens (Eds.), Chemiluminescence in Analytical Chemistry, Marcel Dekker, New York, NY, 2001, p. 393.
[17] A.M. Garcia-Campaña, W.R.G. Baeyens, N.A. Guzman, in: A.M. Garcia-Campaña, W.R.G. Baeyens (Eds.), Chemiluminescence in Analytical Chemistry, Marcel Dekker, New York, NY, 2001, p. 428.
[18] A.K. Campbell, Chemiluminescence: Principles and Applications in Biology and Medicine, Ellis Horwood/VCH, Chichester/Weinheim, 1988.
[19] S. Daunert, G. Barrett, J.S. Feliciano, R.S. Shetty, S. Shrestha, W. Smith-Spencer, Chem. Rev. 100 (2000) 2705.
[20] A. Keane, P. Phoenix, S. Ghoshal, P.C.K. Lau, J. Microbiol. Methods 49 (2002) 103.
[21] T. Chianéa, Ph.J.P. Cardot, E. Assidjo, J. Monteil, P. Krausz, J. Chromatogr. B 734 (1999) 91.
[22] X. Tong, K.D. Caldwell, J. Chromatogr. B 674 (1995) 39.
[23] T.I. Quickenden, P. Cooper, Luminescence 16 (2001) 251.
[24] T.I. Quickenden, J.I. Creamer, Luminescence 16 (2001) 295.
[25] M. Yamaguchi, H. Yoshida, H. Nohta, J. Chromatogr. A 950 (2002) 1.
[26] D. Melucci, M. Guardigli, B. Roda, A. Zattoni, P. Reschiglian, A. Roda, Talanta 60 (2003) 303.
[27] P. Reschiglian, A. Zattoni, D. Melucci, B. Roda, M. Guardigli, A. Roda, J. Sep. Sci 26 (2003) 1417.
[28] J.P. Cardot, J.-M. Launay, M. Martin, J. Liq. Chromatogr. Related Technol. 20 (1997) 2543.
[29] R. Parsons, V. Yue, X. Tong, P. Cardot, A. Bernard, J.P. Andreux, K.D. Caldwell, J. Chromatogr. B 686 (1996) 177.
[30] A. Bernard, B. Paulet, V. Colin, P.J.P. Cardot, TrAC, Trends Anal. Chem. 14 (1995) 266.
[31] R. Chantiwas, R. Beckett, J. Jakmunee, I.D. McKelvie, K. Grudpan, Talanta 58 (2002) 1375.
[32] G. Salvioli, A. Mambrini, R. Lugli, R. Bertolani, E. Zirilli, in: L. Barbara (Ed.), Recent Advances in Bile Acid Research, Raven Press, New York, NY, 1985, p. 17.
[33] P. Reschiglian, A. Zattoni, G. Torsi, D. Melucci, Rev. Anal. Chem. 20 (2001) 239.
[34] G. Torsi, G. Chiavari, C. Laghi, A.M. Asmundsdottir, F. Fagioli, R. Vecchietti, J. Chromatogr. 482 (1989) 207.
[35] D. Melucci, A. Zattoni, S. Casolari, M. Reggiani, R. Sanz, P. Reschiglian, J. Liq. Chromatogr. Related Technol. 25 (2002) 2211.
[36] S.K. Ratanathanawongs-Williams, J.C. Giddings, in: M.E. Schimpf, K.D. Caldwell, J.C. Giddings (Eds.), Field-Flow Fractionation Handbook, Chapter 21, Wiley–Interscience, New York, NY, 2000.
[37] D.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Michotte, L. Kaufman, Chemometrics: a Text Book, Elsevier, New York, 1988, p. 80.