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Journal of Chromatography B, 860 (2007) 173–179
JOURNAL OF CHROMATOGRAPHY B
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Virus analysis by electrophoresis on a microfluidic chip

Victor U. Weiss [a], Viliam Kolivoška [a,b], Leopold Kremser [a], Bohuslav Gaš [b], Dieter Blaas [c], Ernst Kenndler [a,*]

[a] *Institute for Analytical Chemistry, University of Vienna, Vienna, Austria*
[b] *Department of Physical and Macromolecular Chemistry, Charles University, Prague, Czech Republic*
[c] *Max F. Perutz Laboratories, Medical University Vienna, Vienna, Austria*

Received 30 July 2007; accepted 17 October 2007
Available online 19 November 2007

Abstract

Exploiting the advantages of miniaturization of analytical devices we worked out conditions for the analysis of viruses, subviral particles, and virus–receptor complexes on microfluidic chips. To allow for detection via laser-induced fluorescence, the viral capsids were labelled with the fluorescent dye Cy5. We analyzed human rhinovirus serotype 2 and subviral particles, followed the complexation of the virus with a synthetic fragment of the VLDL-receptor, and tracked the heat-induced conversion of intact virions into empty capsids. In contrast to fused silica capillaries, the glass micro-channels allowed for electrophoresis of the analytes without detergent, and analyses were accomplished within few tens of seconds. This opens the avenue towards the analytics of membrane-enveloped viruses and other biological assemblies that are not stable in the presence of detergent. The chip format has the additional advantage of containment and easy disposal, making it particularly attractive for the analysis of infectious material.

Keywords: Chip electrophoresis; Fluorescence; Virus; Cy5; HRV2; VLDL-receptor; Concatemer; Lab-on-a-chip

1. Introduction

Using human rhinoviruses (HRVs) as a model system with low pathogenic potential, we have demonstrated the great utility of capillary electrophoresis for the identification of different viral serotypes and subviral particles, for measurements of the bioaffinity towards antibodies and receptor fragments, and for mimicking the cell attachment by the use of receptor-decorated artificial cell membranes represented by liposomes in a number of papers published in the last few years [1–15]. These viruses, the main cause of common colds, are icosahedral in shape with a diameter of about 30 nm. They are composed of 60 copies each of the four capsid proteins VP1 through VP4. The viral shell encases a genomic single-stranded RNA molecule of roughly 7100 bases. So far 99 HRV serotypes are known that are divided into a major (87 serotypes) and a minor (12 serotypes) receptor group [16–21]. This classification is based on the use of either intercellular adhesion molecule 1 (ICAM-1) or members of the low-density lipoprotein receptor (LDLR) family for cell attachment. The present investigation deals with HRV2 that belongs to the minor receptor group and thus binds very-low-density lipoprotein receptor (VLDLR).

As the virus capsid consists of proteins, it exhibits a ζ-potential in solutions of the appropriate pH and thus moves in an electric field. This makes viral particles and their constituents accessible to electrophoretic analysis. However, a general problem in virus analytics is the large size of the analyte that in most cases precludes the use of gel matrices. We thus used conventional CE in fused silica capillaries.

Although the separation principles are the same in the narrow tube of CE and in the channel in micro-chip electrophoresis, porting the conditions is not trivial. This is even more the case when using a commercial micro-chip instrument, which is conceived for routine analysis under prescribed conditions rather than for method development. In analytical CE instruments the injection method (pressurized, electrokinetic), the dimension and material of the capillary, the kind of the capillary surface (coated or uncoated), the modification or suppression of the electroosmotic

* Corresponding author. Tel.: +43 1427752305; fax: +43 142779523.
E-mail address: ernst.kenndler@univie.ac.at (E. Kenndler).

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doi:10.1016/j.jchromb.2007.10.026

flow, the selection of the voltage and its polarity, etc. can all be chosen at will. These parameters are decisive for separation but are often pre-defined in commercial instrumentation for micro-chip electrophoresis.

Hundreds of papers have been published on electrophoresis on a chip, especially of RNA, DNA, and proteins, in a few cases also of cells and organelles (see, e.g. recent reviews on this topic [22–43]). Grom et al. describe electrohydrodynamic flow and dielectrophoresis in micro-devices for the accumulation and trapping of Hepatitis A virus particles [44] and Vegvary and Hjertén run intact Semliki Forest virus in a hybrid micro-device [45]. However, no work has been published so far for analysis of viruses or subviral particles despite the well-known advantages of chip electrophoresis (shorter analysis time, less consumption of chemicals and solvents, etc.) over conventional CE. It was thus the goal of the present paper to work out conditions for micro-chip electrophoresis of virus and subviral particles based on our experience in conventional CE.

2. Experimental

2.1. Instrumentation

Analyses were carried out on the Agilent 2100 Bioanalyzer system using so-called DNA chips (Agilent Technologies, Waldbronn, Germany) made from soda lime glass. The instrument permitted the analysis of up to 12 samples per chip. It was equipped with a red laser (maximum at λ_{ex} 630 nm) and a blue LED (maximum at λ_{ex} 470 nm). Data were collected and analyzed with Agilent 2100 Expert software.

2.2. Reagents

Human rhinovirus serotype 2 (HRV2) was prepared as described [46]; its purity and concentration were determined as in [1]. The concentration of the HRV2 stock solution was 1.9 mg/mL virions in 50 mmol/L HEPES buffer (pH 7.5). The recombinant concatemer of four copies of repeat 3 of the very-low-density lipoprotein receptor including a hexa-his tag at its C-terminus and a maltose-binding protein at its N-terminus (MBP-V(4 × 3)) was prepared as detailed in [47,48] and used at a concentration of approximately 2 mg/mL. Proteinase K (18 mg/mL in water) from the Quiagen Tissue Kit 12/G7 was used as a 1:10 dilution in water.

Boric acid of analytical grade and sodium hydroxide were obtained from E. Merck (Darmstadt, Germany), sodium dodecyl sulfate (SDS, 99%) was purchased from Sigma Aldrich (Steinheim, Germany), and sodium bicarbonate of analytical grade was obtained from Fluka (Buchs, Switzerland). Cy5 and Sephadex G-100 were from Amersham Bioscience (Little Chalfont, England). Cy5 was diluted in DMSO to yield a stock solution of approximately 25 mmol/L.

2.3. Buffers

Separations were carried out in 100 mmol/L borate buffer, pH 8.3, with different SDS concentrations. For HRV2 labelling 100 mmol/L carbonate buffer, pH 9.1 was used. Size exclusion chromatography (SEC) was carried out employing 50 mmol/L borate buffer, pH 8.3 as eluent. Respective pH values of the buffers were adjusted from boric acid and $NaHCO_3$, respectively, by adding NaOH solution. Cy5 stock solution diluted $1{:}4 \times 10^5$ in 100 mmol/L borate buffer (pH 8.3) was employed for detector adjustment. Prior to usage, buffers were spin filtered for 10 min at 5200 rpm (Centrifuge 5415D, Eppendorf, Hamburg, Germany) through Corning Spin-X centrifuge tube filters (cellulose acetate membrane, non-sterile, pore size 0.22 μm, obtained from Sigma Aldrich).

2.4. Chip handling

Agilent DNA Chips were filled with 12 μL BGE on the Agilent Chip Priming Station for 20 s, but else according to the recommendations of the manufacturer. Twelve microliters of the respective BGE were put into inlet and waste well (numbered A4 and B4 analogous to Ref. [49]), 12 μL of borate buffer containing Cy5 into well D4. Samples (6 μL) were put into the sample wells (A1–D3). The electrodes of the instrument were cleaned after the replacement of each chip with an electrode cleaner chip filled with double distilled water.

The script provided by Agilent for DNA analysis had to be adapted prior to our separations. The script is usually not known by the operator, who chooses an assay for application. The part of the assay, which defines the operational steps during a chip run, is called script. For the present analysis the script was modified such that for the respective outlet wells (B4 and D4 for injection, C4 for electrophoretic runs) lower potentials were set than for the respective inlet wells (A1 to D3 for injection, A4 for electrophoretic runs). This corresponds to positive polarity mode as usually employed in CE analysis of HRV2. Samples were injected electrokinetically into the separation channel of the DNA Chip by application of 1300 V, the separation voltage was 800 V (approx. 19 kV/m). The chip temperature was adjusted to 30 °C during analysis.

2.5. Procedures

Cy5 labelling of HRV2 was carried out as described for other dyes (fluorescein isothiocyanate, FITC [8], and for Cy3.5 [13]) by mixing virus stock solution (5.0 μL), diluted with 100 mmol/L carbonate buffer (pH 9.1), with a 9.0×10^3 (0.4 μL) or a 4.5×10^3 (0.2 μL) fold molar excess of Cy5, respectively. The total volume of the staining mixtures valued 20 μL. The reaction mixtures were vortexed and left overnight at ambient temperature in the dark. Labelled virus was separated from unreacted material as described [8] on self-packed 1 mL filtration tubes (57240-U, Supelco, Bellefonte, PA, USA) packed to approximately 3.5 cm bed height with Sephadex G-100 equilibrated in 50 mmol/L borate buffer (pH 8.3). Fractions of approximately 40 μL were collected and dilutions of 1:12 or 1:15 in 100 mmol/L borate buffer, pH 8.3 were subjected to analysis.

To study the temperature-dependency of the conversion of native virus into subviral particles, virus samples (1:12 diluted

SEC fractions in 100 mmol/L borate buffer, pH 8.3) were incubated at 37, 40, 43, 45, and 48 °C in a water bath under light protection. For complete disassembly of virions into viral proteins and RNA, fractions were heated to 56 °C for 10 min as described [8] after 1:12 dilution in 100 mmol/L borate buffer, pH 8.3, containing 3.1 mmol/L SDS.

Proteolytic digestion was carried out as described [8] by incubating 9.8 μL of sample containing disassembled virions with 0.2 μL of Proteinase K (1.8 mg/mL in water) for 40 min at 37 °C under light protection. For comparison, intact stained virus was incubated with Proteinase K under the same conditions.

Receptor-binding experiments were carried out by mixing undiluted HRV2 samples (0.8 μL of an SEC fraction containing about 6.2 nmol/L labelled virus) with 0.2, 0.3, 0.4, and 0.5 μL of MBP-V(4 × 3) solution, respectively [8]. The resulting mixtures were diluted with 100 mmol/L borate buffer (pH 8.3) to a total volume of 9.6 μL and incubated for 10 min at ambient temperature prior to analyses.

3. Results and discussion

The instrument used in this work is equipped besides a blue LED (λ_{ex} of 470 nm) with a LIF detector operated by a red laser at λ_{ex} of 630 nm. We selected the LIF detector coupled to the red laser for detection, therefore our analytes needed to be labelled with a suitable fluorescent dye prior to analysis. In previous work, we derivatized HRV2 and HRV14 with FITC, sulfosuccinimidyl-7-amino-4-methylcoumarin-3-acetic acid (AMCA-S), and Cy3.5 (a cyanine compound), respectively, to allow for high sensitivity detection. These chemicals react with amino groups of the capsid proteins, most probably with the ε-amino groups of surface-accessible lysines. However, due to their spectral properties these compounds are not suited for excitation with the red laser of the instrument, therefore Cy5, another cyanine compound with the more appropriate excitation/emission wavelengths of 649/670 nm, was employed in the following studies.

3.1. Selection of separation conditions

Cy5 possesses one net negative charge from a sulfate group in excess over its other charges. With the BGE adjusted to pH 8.3, as normally used for HRV analysis, the amino groups of the exposed lysines are protonated ($pK_a = 9.5$); upon derivatization, this positive charge is lost and the virus gains two net negative charges per reacted amino group. Based on the assumption of 240 surface-accessible lysines for the native virion (see, e.g. Ref. [9]), derivatization is to be expected to change the electrophoretic properties of the virus drastically as it becomes more negative. In addition, at high degrees of derivatization, the overall hydropathy of the capsid is also expected to be modified. This has turned out to have important consequences on the interaction with detergents (in particular with SDS) that were indispensable for reliable separations in nearly all CE analyses in fused silica capillaries [50]. In the absence of detergents the virus tended to give distorted peaks and/or spikes due to aggregation and wall adsorption. Indeed, under these circumstances the electrophoretic mobility of HRV2 is mainly determined by its interaction with SDS rather than by its original ζ-potential.

In contrast to CE in fused silica capillaries, in the micro-chip a series of exploratory experiments demonstrated that it was possible to work with a BGE without detergent. The electropherograms of two virus samples (derivatized with a 4.5×10^3 and a 9.0×10^3 fold molar excess of Cy5 over virus, respectively) are presented in Fig. 1. The peak with the longest migration time corresponds to excess dye still present after purification by SEC in this fraction. Fractions still containing free dye were chosen deliberately to evolve separation conditions for all constituents of the staining mixture. In contrast to the fractions presented in Fig. 1, fractions with the highest virus concentrations were dye-free after SEC.

The faster migrating zone in Fig. 1 comprises the only partly resolved peaks of the labelled virus and a contamination of unknown nature, which was found to become heavily labelled with amino-reactive dyes and is present in all virus preparations at different concentrations. The repeatability of the separation is demonstrated by the electropherograms obtained with two different chips (compare the records of the upper with those of the lower panel). Thus, chip electrophoresis enables analysis of HRV2 without detergent although the separation from the contaminant is poor.

SDS not only stabilizes the virus in CE, but it also influences drastically the electrophoretic mobility of derivatized as well as non-derivatized virus [13]. Therefore, different SDS concentrations were assessed for their influence on the separation selectivity for the analytes: virus, contaminant, and residual dye (Fig. 2). Addition of 2 mmol/L SDS does not significantly affect the separation when compared to detergent-free BGE; HRV2 and the contaminant are still co-migrating. At 10 mmol/L SDS, the migration time of HRV2 is drastically shifted and the virus is well separated from the contaminant. However, under this condition, the virus co-migrates with Cy5. Variation of the SDS concentration within these extremes enabled us to find an SDS concentration at which all analytes were baseline separated. The electropherogram at SDS concentration of 3.1 mmol/L gives the best separation; it is shown in Fig. 2, middle panel. Further analyses were carried out at this SDS concentration. It is of note that neither the contaminant nor the free Cy5 changed their relative peak positions as a function of the SDS concentration. This underscores that SDS preferentially interacts with the virus.

3.2. Analysis of subviral particles

Upon heating to a temperature between 50 and 56 °C for some minutes, native (non-labelled) HRV2 looses VP4 and the genomic RNA under formation of subviral empty capsids (also termed 80S- or B-particles). We investigated this conversion in Cy5-labelled HRV2 upon incubation at temperatures between 37 and 48 °C for up to 15 min (in borate buffer without SDS) followed by analysis on the chip. It is of note that the examined SEC fraction did not contain any free dye. Typical electropherograms obtained after exposure to 45 °C for various times are shown in Fig. 3. The virus peak decreases in a time-dependent manner

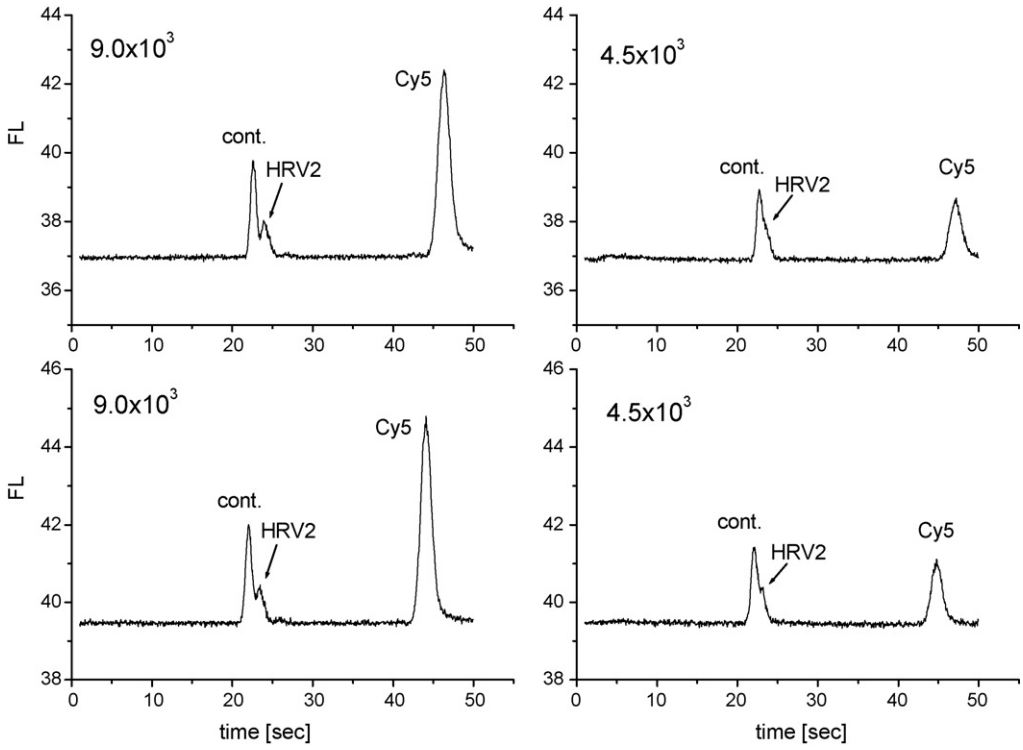

Fig. 1. Electropherograms of HRV2 labelled with Cy5 at 4.5×10^3 or 9.0×10^3 fold molar excess, respectively, run on two different chips (upper and lower panels). Samples were purified by SEC prior to analysis. Fractions still containing free dye after purification were investigated. BGE: 100 mmol/L borate buffer, pH 8.3 without detergents as additives. Sample was diluted 1:15 with BGE. Separations were carried out at 800 V (approx. 19 kV/m). cont.: contaminant; FL, fluorescence signal at $\lambda_{ex}/\lambda_{em}$ 630/680 nm.

with the concomitant appearance and subsequent increase of a broader peak at longer migration time. This peak must correspond to the subviral 80S-particle or to the individual viral proteins because only these species exhibit the fluorescence labels.

3.3. Kinetics of denaturation

As shown above, incubation of HRVs at 45 °C results in the generation of empty capsids. We then assessed whether the temperature- and time-dependent structural changes of the virus could be followed by the chip-based instrument. The peak area, A_{cont}, of the contaminant present in all virus preparations (see above) was taken as a kind of an internal standard. The area of the Cy5-labelled virus peak (A_{virus}) was related to A_{cont} and measured as function of the incubation time at different temperatures (Fig. 4). We normalized this ratio to that at incubation time zero (A^0_{virus}/A^0_{cont}) according to $(A_{virus}/A_{cont})/(A^0_{virus}/A^0_{cont})$. At 37 °C the relative virus peak area remained unchanged during the entire incubation time, at 40 °C a slight decrease of the relative peak area was found. From 43 °C onwards a clear exponential decay of the peak area with incubation time is seen; this decay accelerated with an increase in temperature. At 48 °C, the virus peak vanishes within 3 min, which is in sharp contrast to the behavior of the unlabelled virions [7]. Stability was expressed by a time constant, τ, of the conversion, which was derived by fitting the data to an exponential decay of first order. A first-order kinetics is evident because the virus thermally decomposes without a reaction partner, and it is most unlikely that the decomposition products recombine. For 43, 45, and 48 °C the time constants derived were 4.9, 2.4, and 0.38 min, respectively. Interestingly, unlabelled virions converted with a time constant of about 4 min at 55 °C [7]. Therefore, it can be concluded that derivatization resulted in a marked loss of thermostability. Furthermore, it follows that the chip-based instrument enables the measurement of the thermal stability of virus particles as a function of the covalent modification of surface-exposed lysines by the fluorescent label.

3.4. Proteolysis of viral capsid proteins

Complete disintegration of the virus into its capsid proteins (and its genomic RNA) takes place upon heating in the presence of SDS. An electropherogram of highly purified Cy5-labelled virus (excess of dye was removed via SEC) incubated for 10 min at 56 °C in borate buffer, pH 8.3, containing 3.1 mmol/L SDS is shown in Fig. 5. Comparison of panel (A) with panel (B) shows that the virus peak completely disappeared and a new peak appeared. Based on our experience we tentatively attributed this peak – in panel (B) – to labelled capsid proteins. The peak might either correspond to VP1 alone, as it exhibits the most exposed lysines and thus is most strongly labelled, or include the other capsid proteins as well. To confirm that the peak originates from protein, the sample was subjected to proteolytic digestion with Proteinase K (at 37 °C for 40 min). Indeed the

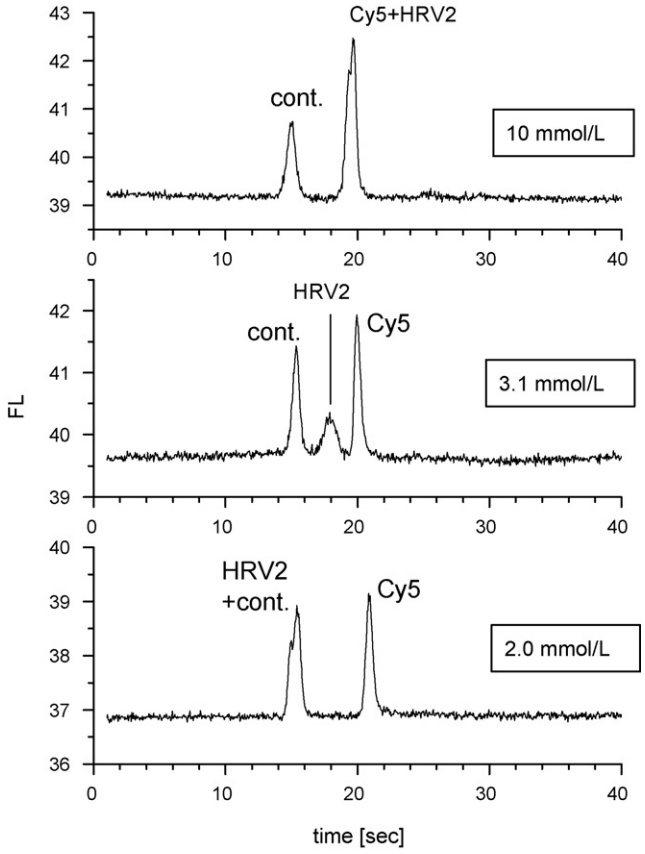

Fig. 2. Separation of HRV2, Cy5, and contaminant (cont.) obtained at 2.0, 3.1, and 10 mmol/L SDS in the BGE. Ratio of Cy5 to virus in the reaction mixture was 4.5×10^3. Separations were carried out at 800 V (approx. 19 kV/m). Samples were purified by SEC prior to analysis and diluted 1:15 in 100 mmol/L borate buffer, pH 8.3. FL, fluorescence signal at $\lambda_{ex}/\lambda_{em}$ 630/680 nm.

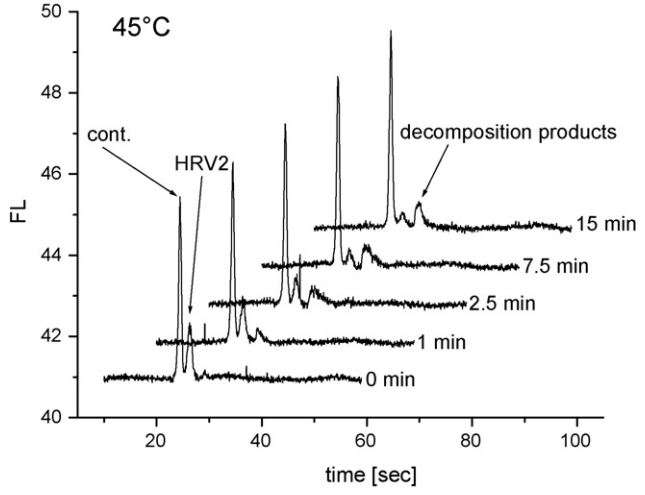

Fig. 3. Conversion of Cy5-labelled HRV2 induced by incubation at 45 °C under light protection for 0 min, 1 min, 2.5 min, 7.5 min and 15 min. Separations were carried out at 800 V (approx. 19 kV/m). Samples were diluted 1:12 in 100 mmol/L borate buffer, pH 8.3. BGE for separation: 100 mmol/L borate buffer, pH 8.3, containing 3.1 mmol/L SDS. Staining was with 9.0×10^3 fold molar excess of dye. FL, fluorescence signal at $\lambda_{ex}/\lambda_{em}$ 630/680 nm.

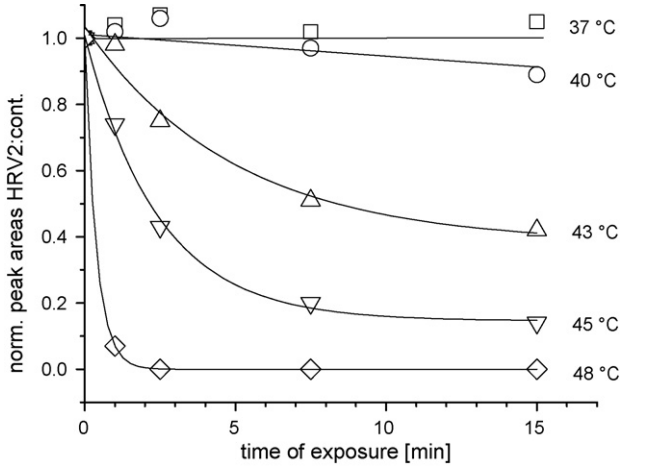

Fig. 4. Change of peak area of Cy5-labelled HRV2 upon incubation at different temperatures for different times. The peak areas of HRV2 were related to that of the contaminant and set to 1 at time zero. Data are the average from duplicate measurements with a repeatability of typically 5–10%.

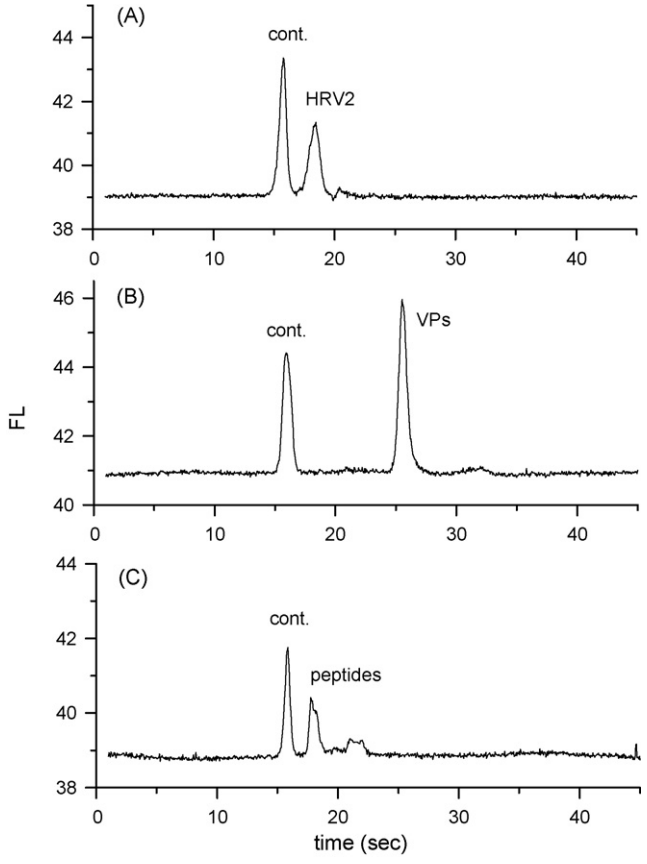

Fig. 5. Electropherograms of (A) Cy5-labelled HRV2 diluted 1:12 in 100 mmol/L borate buffer, pH 8.3; (B) of viral proteins formed upon heating of (A) to 56 °C for 10 min in the same buffer but containing 3.1 mmol/L SDS; and of (C) after proteolytic digestion of (B). Separations conditions as in Fig. 3. Staining was with 4.5×10^3 fold molar excess of dye; this SEC fraction did not contain free Cy5. FL, fluorescence signal at $\lambda_{ex}/\lambda_{em}$ 630/680 nm.

peak in the electropherogram disappeared and a number of smaller peaks arose that correspond to the labelled peptides (Fig. 5C).

3.5. Bioaffinity reaction with receptor fragment

We used the present micro-device to analyze the formation of complexes between HRV2 and a recombinant soluble concatemer of module 3 of the human very-low-density lipoprotein receptor (MBP-V(4 × 3)). Natural VLDL-receptor possesses eight binding modules (V1–V8). The present receptor fragment is a concatemer consisting of 4 identical copies of V3 connected in tandem; at the N-terminus it carries an MBP and at the C-terminus a hexa his-tag. Structures derived by cryo-electron microscopy and X-ray crystallography showed that single V3 modules attached to the BC and HI loop of VP1 close to the fivefold axis of icosahedral symmetry [46,51,52].

The binding assays were carried out with HRV2 at a constant concentration of 520 pmol/L and at final receptor concentrations of 0.667, 1.00, 1.33, and 1.67 μmol/L. The components were incubated at ambient temperature for 10 min and then analyzed (Fig. 6). Upon addition of MBP-V(4 × 3) the initial virus peak was shifted towards longer migration times, a clear indication for complex formation. Moreover, the peak splitted into a number of poorly resolved peaks, which can be tentatively related to multiple virus complexes with the virus differently occupied with receptors. Increasing the receptor concentration led to sharper peaks because a homogenous saturated complex was formed.

These effects can be better visualized when plotting the mobility as function of the excess of ligand, as shown in Fig. 7. The length of the bar represents the peak width (measured at half height) corresponding to virus and complexes; in case of a set of poorly resolved peaks the width of whole peak bundle was taken. This representation clearly illustrates that the

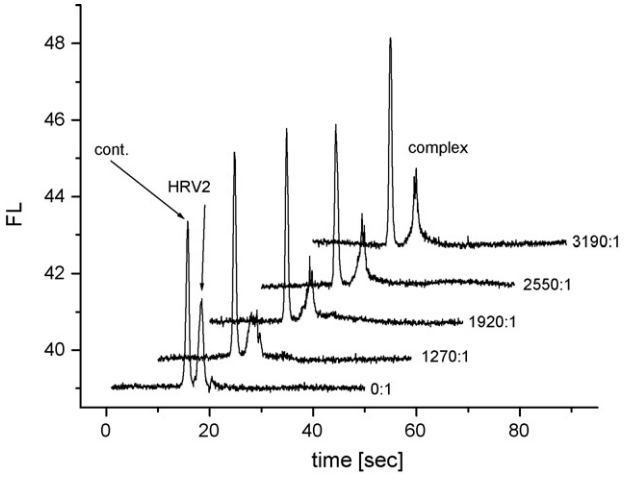

Fig. 6. Electropherograms of complexes between HRV2 and the recombinant soluble artificial receptor MBP-V(4 × 3). Virus was mixed with the receptor at a molar excess of between 0 and 3190 and diluted 1:12 with 100 mmol/L borate buffer, pH 8.3. Separation conditions as in Fig. 3. The virus was labelled with a 4.5×10^3 fold molar excess of dye; this SEC fraction did not contain free Cy5. FL, fluorescence signal at $\lambda_{ex}/\lambda_{em}$ 630/680 nm.

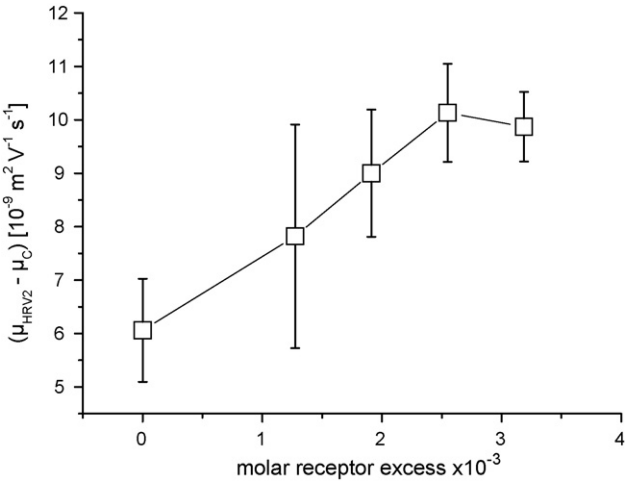

Fig. 7. Mobility shift and width of complex peaks of HRV2 with MBP-V(4 × 3) as function of the molar excess of the receptor. The mobility shift is the difference between the mobility of contaminant and HRV2 (Fig. 6). The lengths of the bars indicate the peak widths (expressed in mobility) measured at halve peak height.

mobility of the complexes increases with increasing receptor concentration and reaches a plateau at high excess indicating the formation of saturated complexes. Furthermore, the length of the bar illustrates that the narrow peak of the virus firstly broadens significantly upon complex formation but its width decreases again upon saturation. The peak presumably corresponding to the saturated complex has a similar width as that of free virus.

The behavior of the virus upon complexation with the receptor fragment is reminiscent of previous work using conventional CE instrumentation. However, there are some differences. Most striking is that the ratio of the molar concentrations of MBP-V(4 × 3) and HRV2, at which complex formation could be monitored (more than 1000-fold), was much higher than observed previously using MBP-V(5 × 3), a pentameric concatemer of V3 (a 20-fold molar excess of receptor fragment resulted in saturation [53]). This might have a number of reasons: (i) MBP-V(4 × 3) possesses only four V3 modules that bind with lower avidity. (ii) Although the receptor was present at higher molar excess, the concentrations of the reactants were by 3 orders of magnitude lower than in previous work (500 pmol/L vs. several tenth of μmol/L) resulting in higher dissociation of the complex; (iii) derivatization of the exposed lysines with Cy5 must weaken the interaction since the lysines are intimately involved in the interaction with the receptor.

4. Conclusions

Micro-chip electrophoretic analysis in a commercial device equipped with a red laser for LIF detection was carried out for separation of Cy5-labelled HRV2 and subviral particles. Complexation of the virus with a synthetic fragment of the VLDL-receptor was assessed via the shift of the migration time of the virus peak as a function of excess receptor and the concomitant change in the peak shape. Conversion of intact virions into subviral particles could be followed by the detection of

empty capsids, and the kinetics of denaturation was measured as a function of temperature and time.

The micro-device has two advantages when compared to conventional capillary electrophoresis: analysis time was within tens of seconds instead of tens of minutes and in principle there was no need to add detergents to the BGE. The first point is relevant for routine analysis of large sample numbers, the second point is beneficial for the analysis of membrane-bounded organelles, enveloped viruses, and liposomes.

Acknowledgements

This work was supported by the Austrian Science Foundation (project P19365) and by a research grant from Agilent Technologies Inc., Santa Clara, CA (grant A-200610). The authors thank the Austrian Exchange Service for a grant for Viliam Kolivoška (CEEPUS network CII-HU-0010-01-0607) and Irene Goesler for the preparation of the viruses. The authors also thank Agilent Technologies GmbH, Waldbronn for supporting script modifications on the Bioanalyzer instrument.

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