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Research Article

Electrophoresis on a microfluidic chip for analysis of fluorescence-labeled human rhinovirus

We report the analysis of human rhinovirus serotype 2 (HRV2) on a commercially available lab-on-a-chip instrument. Due to lack of sufficient native fluorescence, the proteinaceous capsid of HRV2 was labeled with Cy5 for detection by the red laser (λ_{ex} 630 nm) implemented in the instrument. On the microdevice, electrophoresis of the labeled virus was possible in a BGE without stabilizing detergents, which is in contrast to conventional CE; moreover, analysis times were drastically shortened to the few 10 s range. Resolution of the sample constituents (virions, a contaminant present in all virus preparations, and excess dye) was improved upon adaptation of the separation conditions, mainly by adjusting the SDS concentration of the BGE. Purity of fractions from size-exclusion chromatography after labeling of virus was assessed, and affinity complex formation of the labeled virus with various recombinant very-low-density lipoprotein receptor derivatives differing in the number of concatenated V3 ligand binding repeats was monitored. Virus analysis on microchip devices is of particular interest for experiments with infectious material because of easy containment and disposal of samples. Thus, the employment of microchip devices in routine analysis of viruses appears to be exceptionally attractive.

Keywords:
Chip electrophoresis / Concatemer / Cy5 / HRV2 / Lab-on-a-chip / Virus
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1 Introduction

The use of a narrow open tube as separation channel for electrophoresis enables direct analysis of large biological assemblies like viruses, bacteria, cells, and organelles in free solution. These particles range from several tens of nanometres to some few micrometers in size, and usually cannot be electrophoresed in the presence of stabilizing matrices like gels, due to the small dimensions of the pores of these materials. These biological particles possess a ζ-potential and move therefore in solution in colloidal or subcolloidal form under the influence of an electric field applied along the separation capillary. As the cross-section area of the separation channels of microchips has the same order of magnitude as those of the capillaries in CE but the path is much shorter, these microdevices allow for much shorter analysis times but also exhibit less consumption of chemicals and solvents (whereas not smaller sample volumes) and have the additional advantage that the whole chip is disposed after the run thus minimizing contamination.

In the context of virus analysis, microdevices were rather used for the separation of viral constituents than for electrophoresis of intact viruses. For example, chip electrophoresis of viral RNA, or DNA produced *via* PCR, served for genotyping Hepatitis B [1] and Hepatitis C virus [2], for the detection of Dengue-2 virus [3], of the Coronavirus causing severe acute respiratory syndrome (SARS) [4], for analysis of DNA samples of Cytomegalovirus [5] and Herpes simplex virus [6]. Only in a few cases, the viruses themselves were subjected to electrophoresis; *e.g.* electrohydrodynamic flow and dielectrophoresis in microdevices were applied for the accumulation and trapping of Hepatitis A virus particles [7] and Vegvari and Hjerten [8] run intact Semliki Forest virus in a hybrid microdevice.

In the last years, we have developed methodologies based on CE for the identification of human rhinovirus (HRV) and derived subviral particles, for measuring the bioaffinity to-

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Abbreviations: **FL,** fluorescence; **FU,** fluorescence units; **HRV2,** human rhinovirus serotype 2; **MBP,** maltose binding protein; **SEC,** size-exclusion chromatography; **VLDLR,** very low density lipoprotein receptor

wards antibodies and receptor fragments, or assessed the attachment of HRVs to receptor-decorated artificial cell membranes represented by liposomes [9–23]. It was the goal of the present paper to investigate the possibility of porting these methodologies from the capillary to the microchip format in order to exploit the latter's advantages. As the applied instrumentation (a commercial microdevice) utilized a red laser at λ_{ex} 630 nm for high-sensitivity detection, virus particles were fluorescence (FL)-labeled prior to analysis and preseparated from the excess of dye by size-exclusion chromatography (SEC).

2 Materials and methods

2.1 Chemicals

The fluorescent dye Cy5, and Sephadex G-100 were obtained from Amersham Bioscience (Little Chalfont, England); Cy5 was solved in DMSO (approximately 25 mM) as stock solution. Boric acid and sodium hydroxide (both analytical grade) were purchased from E. Merck (Darmstadt, Germany), SDS (99%) was from Sigma–Aldrich (Steinheim, Germany) and sodium bicarbonate (analytical grade) from Fluka (Buchs, Switzerland). Water was doubly distilled from a quartz apparatus.

2.2 Biological materials

Human rhinovirus serotype 2 (HRV2) was prepared and its purity and concentration was checked as described in ref. [9, 24]. Experiments aimed at adjusting separation selectivity were carried out with labeled virus from a 5.0 μL aliquot of a virus batch with a concentration of 1.9 mg/mL in 50 mM HEPES buffer (pH 7.5). For complex formation with receptor fragments a 2.5 μL aliquot of an HRV2 preparation with a concentration of 5.0 mg/mL was used for the according labeling procedure. Concatemers of module 3 (V3) of the very-low-density lipoprotein receptor (VLDLR) with one, two, three, four, five or seven copies fused to maltose binding protein (MBP) at their *N*-terminus and a hexa-his tag at their *C*-terminus were prepared as described in detail in ref. [25, 26]. The concentration of the recombinant concatemers was approximately 2.0 mg/mL in TBS buffer (20 mM Tris-HCl, 150 mM NaCl, additionally containing 20 mM $CaCl_2$) each.

2.3 Instrumentation

Analyses were carried out on the Agilent 2100 Bioanalyzer system (Agilent Technologies, Waldbronn, Germany). The instrument was equipped with a blue light-emitting diode (LED) (λ_{ex} 470 nm) and a red laser (λ_{ex} 630 nm). As in our previous paper [27], peak monitoring was exclusively carried out with the red laser. Commercially available DNA Chips from Agilent were employed. Data were collected and analyzed with Agilent 2100 Expert software. All solutions were centrifuged on a tabletop centrifuge (model 5415D, Eppendorf, Hamburg, Germany) prior to use.

2.4 Chip handling

Chips were handled as described [27] following the instructions of the manufacturer on the Agilent Chip Priming Station with settings for DNA Chips. Twelve microliters instead of nine microlitres, as recommended by the Agilent Kit Guide (DNA Analysis) of the respective BGE, were pressurized for 20 s (at approx. 4 bar) from the outlet well C4 (numbering of wells analogous to ref. [27]). Then, 12 μL of the respective BGE were added to inlet (A4) and waste well (B4), and 12 μL of 100 mM borate buffer containing Cy5 (pH 8.3, Cy5 diluted to 63 nM) for detector adjustment to well D4. Six microlitres of each sample were added to the remaining 12 wells A1-D3. Prior to analysis with each chip, the electrodes of the instrument were cleaned by the aid of the Electrode Cleaner Chip filled with 350 μL doubly distilled water. Changes in the script for DNA analysis were accomplished as described [27] and allowed to run the analyses in the presence of electroosmosis. The script defines the operational steps during a chip run.

2.5 Buffer preparation

Separations were carried out in 100 mM borate buffer containing 0.0, 1.0, 2.0, 2.8, 3.1, 3.7, 5.0 and 10 mM SDS, respectively. These BGEs were prepared in double distilled water and the pH of all buffers was adjusted to 8.3 with NaOH. Aliquots of these buffers were spin-filtered for 10 min at 5200 rpm through Corning Spin-X centrifuge tube filters (cellulose acetate membrane, nonsterile, pore size 0.22 μm, Sigma–Aldrich). SEC was carried out in borate buffer (50 mM, pH 8.3, without SDS). For labeling the virus, 100 mM carbonate buffer was prepared in doubly distilled water, its pH was adjusted to 9.1 with NaOH, and it was filtered through 0.20 μm cellulose acetate membrane syringe filters (sterile Minisart NML, Sartorius, Göttingen, Germany).

2.6 Procedures

FL labeling of HRV2 was carried out as described [27] by mixing 5.0 μL virus solution at 1.9 mg/mL (molecular mass of HRV is 8.5 MDa) with 14.6 μL carbonate buffer (100 mM, pH 9.1) and 0.4 μL Cy5 solution. This resulted in a 9.0×10^3-fold molar excess of dye over virus. Labeling of HRV2 (2.5 μL aliquots of the preparation with 5.0 mg virus *per* mL) with a 5.2×10^3-fold molar excess of Cy5 (0.3 μL solution) was done accordingly. The reacting mixtures were vortexed and incubated under light protection overnight (approx. 18 h) at ambient temperature.

Low-molecular-mass material was removed *via* SEC on Sephadex G-100 in 50 mM borate buffer (pH 8.3) as described [16] on self packed columns with a bed height of ap-

proximately 3.5 cm (using 1 mL filtration tubes and polyethylene frits, both purchased from Supelco, Bellefonte, USA, catalogue numbers 57240 and U57244). Fractions of approximately 40 μL were collected.

Adjustment of the BGE with respect to the SDS concentration was carried out with samples from SEC fraction 14 (staining ratio $1{:}9.0 \times 10^3$) diluted 1:15 in 100 mM borate buffer (pH 8.3). Virus content of fractions after SEC was analysed prior to complex formation between virus and receptors with 1:12 diluted fractions in 100 mM borate buffer (pH 8.3). Complex formation was carried out with fraction 9 of the HRV2 sample stained with 5.2×10^3-fold molar excess of dye. This fraction contained about 32% of the total labeled virus as determined from the peak area, corresponding to a virus concentration of roughly 12 nM, and was applied in 1:12 dilution (100 mM borate, pH 8.3) for complex formation. Briefly, the respective receptor solutions were diluted to 2.3, 4.7, 7.0 and 9.4 μM in SDS free borate buffer (100 mM, pH 8.3) and 1.0 μL of these solutions were mixed with 6.0 μL of diluted virus fraction 9, yielding molar excess levels of 400, 800, 1200 and 1600 of receptor over virus. Mixtures were vortexed and incubated at ambient temperature for 10 min in the dark prior to analyses.

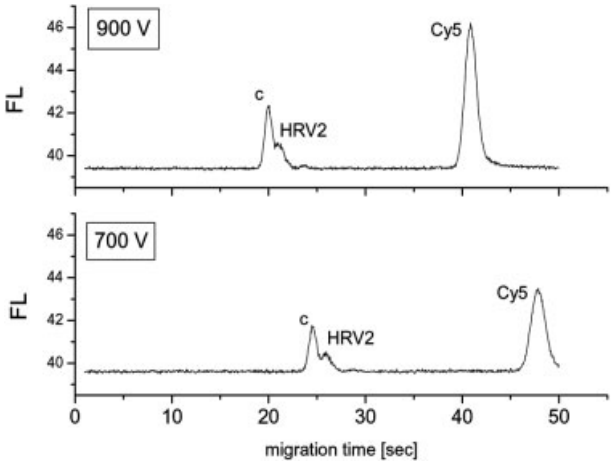

Figure 1. Electropherograms at two different separation voltages of HRV2 labeled with Cy5 with a 9.0×10^3-fold molar excess of dye over virus. Samples were purified by SEC prior to analysis; one fraction still containing free dye was diluted 1:15 with 100 mM borate buffer, pH 8.3 (*i.e.* BGE) without detergents as additives. Separations were carried out at 700 V (approx. 17 kV/m) and 900 V (approx. 22 kV/m) respectively. c, Contaminant. Detection of FL signal at $\lambda_{ex}/\lambda_{em}$ 649/670 nm.

3 Results and discussion

3.1 Labeling of HRV2 with Cy5

HRVs consist of a proteinaceous capsid which encases an RNA genome. Protein as well as the RNA can be labeled with fluorophores. We have already demonstrated labeling of the RNA inside the intact virus by RiboGreen [16], and labeling of capsid proteins by various amine-reacting dyes [17, 18]. As in ref. we here use the cyanine dye, Cy5, which reacts with primary amines under formation of a fluorescent product with λ_{ex} of 649 and λ_{em} of 670 nm, appropriate for detection with the red laser of the instrument.

The icosahedral virus capsid consists of 12 pentamers assembled from four viral proteins, VP1 to VP4. Exposed lysines of VP1 are particularly suited for reaction with the dye. From the 3-D structure of the virion [28], about 240 ε-amino groups might be accessible on the native virion for the reactive dye. However, using FITC we found that only approximately 90 lysines reacted [17]. Based on this finding, reaction with Cy5 would introduce 180 additional negative charges *per* virion. At the given pH of the BGE the derivatized particle should thus possess a higher anionic mobility than the native virus.

Typical electropherograms obtained from a sample of the labeled virus are shown in Fig. 1. BGE was borate buffer, pH 8.3. As the pK_a of borate is 9.25, the buffering capacity of this BGE is still sufficiently high (it is about 20% of the maximum capacity). The electropherograms were recorded at different separation voltages. The peak of the dye which was incompletely removed by SEC in this fraction is detected together with two not fully resolved peaks at lower migration time. The smaller peak originates from the virus; the larger one is from a contaminant of unknown nature that we always detected in the virus preparations. Resolution of these two peaks is influenced by the degree of derivatization: the higher the excess of dye the better is the resolution (not shown). This is seemingly due to the increasing anionic mobility of the labeled virus as mentioned above, which leads to a larger difference in migration time in relation to that of the contaminant. In accordance with earlier experiments [9] the virus peak decreases upon heating of the sample because the virus undergoes structural changes resulting in subviral particles whereas the peak from the contaminant remains at its initial position (data not shown).

It is remarkable that electrophoresis of the virus can be carried out on the glass chip without the addition of detergent to the BGE, which was not possible in the capillary format using fused-silica tubes. In that case, the absence of detergents resulted in formation of aggregates indicated by nonreproducible spikes in the electropherograms, by severe tailing, or even by the absence of any peaks attributable to the injected virus.

3.2 Separation selectivity and SDS concentration

In previous work we found that the mobility of the virus is significantly affected by the anionic detergent SDS present in the BGE whereas the uncharged detergent dodecyl-PEG (D-

PEG, Thesit) was without influence [21]. We thus tried to improve the poor separation of the virus from the contaminant (which can be seen from Fig. 1) by applying SDS at various concentrations. The resulting selectivity coefficients for the two consecutively migrating components are shown in Fig. 2. This coefficient is expressed as the ratio (the larger mobility always being the numerator) of the total mobilities of the respective separands: the virus, the contaminant and Cy5. It is unit at no separation and >1 if the analytes are being separated. We found that at zero or low SDS concentration the selectivity coefficient is very high for the pair HRV2-Cy5, but it is close to 1 for HRV2 and the contaminant. Increasing the SDS concentration results in a steep descent of the curve for HRV2-Cy5 and at SDS concentrations >5 mM the value of the selectivity coefficient approaches 1. In contrast, the curve for the pair HRV2-contaminant increases with the SDS concentration with a sigmoid shape and approaches a plateau with a selectivity coefficient of about 1.2 at more than 5 mM SDS. The two curves subtend at 3.1 mM SDS, which is thus the best compromise for separation. From the electropherograms in this crucial SDS concentration range (we show those between 2.0 and 5.0 mM SDS in Fig. 3) it can be seen that indeed at 2.8 mM SDS, the virus and the contaminant almost comigrate, and at 5 mM the pair HRV2 and Cy5 migrates in a single peak. However, at 3.1 mM HRV2 is baseline-separated from both other constituents; this SDS concentration in the BGE was thus used for all further analyses.

Finally we give some figures of merit. The reproducibility of the migration times of the virus of the same samples but with different chips was typically about 6%. With the contaminant taken as internal standard the relative migration times of the virus peaks varied within less than 3%. This value is also typical for the reproducibility of the peak areas of

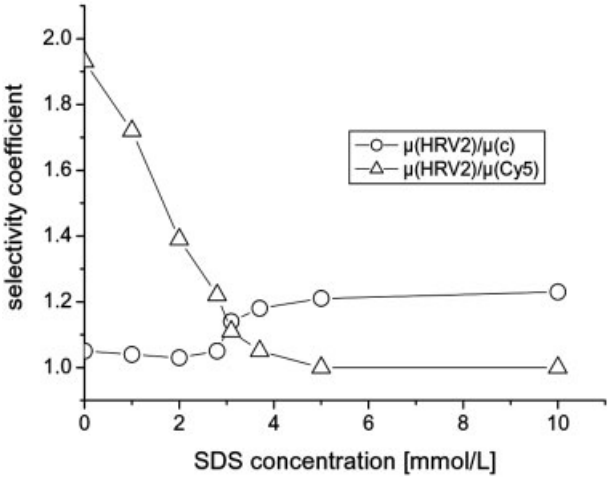

Figure 2. Selectivity coefficients of labeled HRV2 and Cy5 and of labeled HRV2 and the contaminant, c, respectively, as a function of SDS concentration of the BGE. The selectivity coefficient is the ratio of the total electrophoretic mobilities, μ, for each pair of separands.

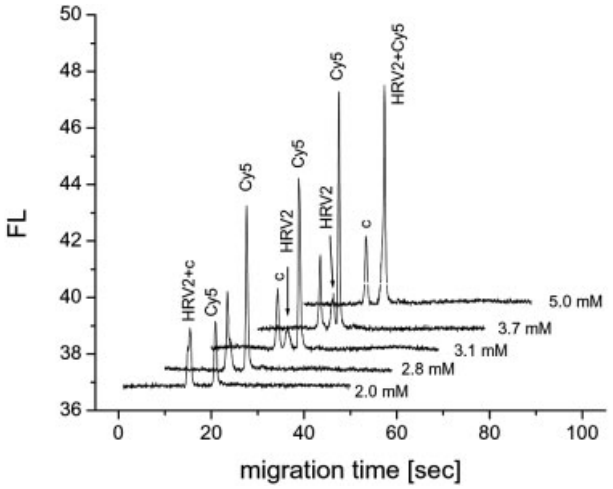

Figure 3. Electropherograms of Cy5-labeled HRV2 at SDS concentrations of between 2.0 and 5.0 mM. Sample preparation was as in Fig. 1. BGE was 100 mM borate buffer (pH 8.3) containing SDS as indicated. Separations were carried out at 800 V (approx. 19 kV/m), the FL signal was recorded at $\lambda_{ex}/\lambda_{em}$ 649/670 nm. c, Contaminant.

the virus at different chips. The sensitivity of the method, expressed for Cy5, was 0.74 FU/nM (FU is a fluorescence unit). The LOD (derived from the three-fold SD of the background noise) was about 100 pM for Cy5 and 20 pM for labeled HRV2.

3.3 Analysis of SEC fractions

Fluorescent labeling of the capsid proteins has to be carried out with a large excess of the dye. Unreacted Cy5 was removed by using a Sephadex G100 column (cut off is 100 kDa). Fractions of 40 μL were collected and analyzed by CE on the chip with runtimes of less than a minute. The electropherograms using a BGE containing 3.1 mM SDS are shown in Fig. 4 and demonstrate the size-dependent chromatographic elution profile of the components. In Fig. 5 the quantitative result is depicted showing the peak areas as bars in dependence on the fraction number. It can be concluded that the chip analysis allows for extremely rapid determination of the fractions enabling to assess the best compromise between virus purity and recovery (fraction 9 and 10 for the presented 5.2×10^3-fold molar excess of dye over virus). It thus appears very well suited for quality monitoring of viral preparations.

3.4 Affinity reactions with receptor fragments

Minor group HRVs specifically bind the low-density lipoprotein receptor (LDLR), VLDLR and the LDLR-related protein (LRP) [24, 29, 30]. LDL-receptors are mosaic proteins; their ligand-binding domains at the *N*-terminus are composed of various numbers of ligand binding repeats, each about 40

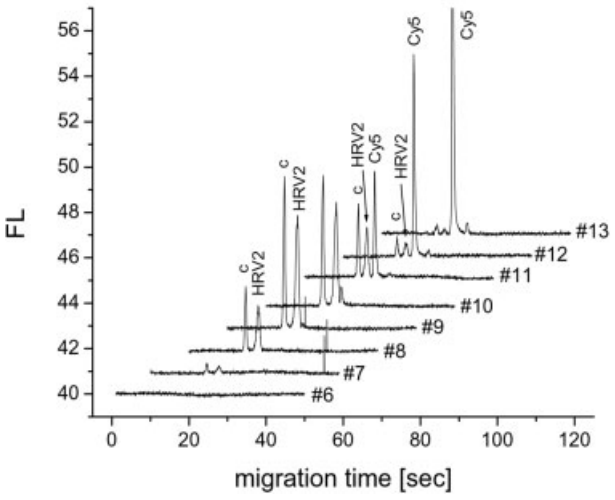

Figure 4. Electropherograms of fractions 6–13 from the SEC separation of labeled HRV2 (at 5.2×10^3-fold molar excess of Cy5) and excess dye. Fractions from SEC were diluted 1:12 in non-SDS containing 100 mM borate buffer (pH 8.3) prior to analysis. Separations were carried out in the same buffer, but containing 3.1 mM SDS. Separation conditions are as in Fig. 3.

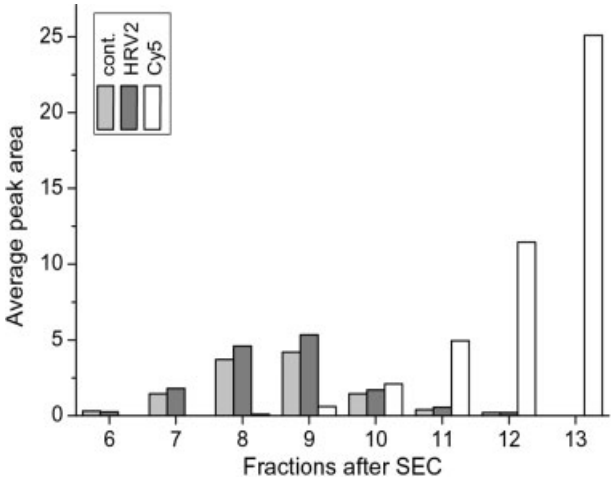

Figure 5. Peak areas (indicated by bars) of HRV2, Cy5 and the contaminant, c, from chip analysis of SEC fractions 6–13 as shown in Fig. 4. Peak areas are in FUs times seconds.

amino acids in length. LDLR has seven such modules, VLDLR has 8, and LRP has clusters of 2, 8, 10 and 11. A number of concatemers of repeat V3 (module number 3 of VLDLR) fused to MBP have been used previously in a number of investigations to determine the interaction parameters [18, 19, 26, 29, 31, 32]. A V3 module attaches to surface loops of VP1 close to the five-fold axis of icosahedral symmetry but concatemers adopt a ring-like conformation winding around the icosahedral vertices, which strongly increases the avidity (Querol-Audi, J., Fita, I., Konecsni, T., Wruss, J. *et al.*, in preparation).

Labeled HRV2 was incubated with MBP-V(3×3) (the concatemer formed by 3 repeats of V3, the module number 3) and the formation of complexes was followed by chip electrophoresis. A typical series of electropherograms obtained upon reaction of the virus with MBP-V(3×3) at various molar ratios is shown in Fig. 6. We have chosen SEC fraction number 9 (nearly no free Cy5 was present) in order to avoid comigration of free Cy5 and the resulting virus–receptor complex. The initially narrow virus peak shifts to longer migration times (*i.e.* to higher effective mobilities counter that of the EOF) upon complexation with the receptor fragment, and broadens due to the formation of multiple complexes. This result is reminiscent of previous investigations of complex formation between MBP-V(5×3) and HRV2 using conventional CE [19, 22, 31]. It shows that complexes of labeled HRV2 with MBP-V(3×3) can be resolved by this type of electrophoresis. However, these are most probably less well-defined because of lower avidity and the possibility of binding *via* three and two modules to the five symmetry-related binding sites [33].

We then analyzed the formation of complexes between a set of concatemers with 1, 2, 4, 5 and 7 repeats of module 3. The complexes show a similar electrophoretic behavior as those of virus and MBP-V(3×3), however, at different molar excess of the receptors over virus. The results are depicted in Fig. 7, where the total mobility of the complexes was related to that of the noncomplexing contaminant that was taken as the internal standard. In all cases, the mobility increased with increasing receptor concentration, and the curve reached a plateau at the highest molar excess of receptor. We noticed that the shape of these curves depends not only on the kind of receptor but also varies with the degree of derivatization of the virus, and even with the individual prepara-

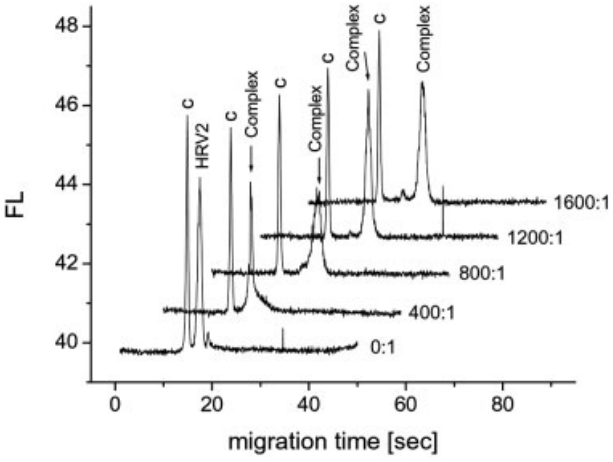

Figure 6. Labeled HRV2 forms a complex with MBP-V(3×3). Virus sample from SEC fraction 9 was incubated with MBP-V(3×3) at molar excess of receptor to virus ranging from 400:1 to 1600:1 as indicated. A virus sample diluted to a respective concentration in 100 mM borate buffer (pH 8.3) without receptor is presented for comparative reasons. For details see text. Separation conditions as in Fig. 3. c, Contaminant.

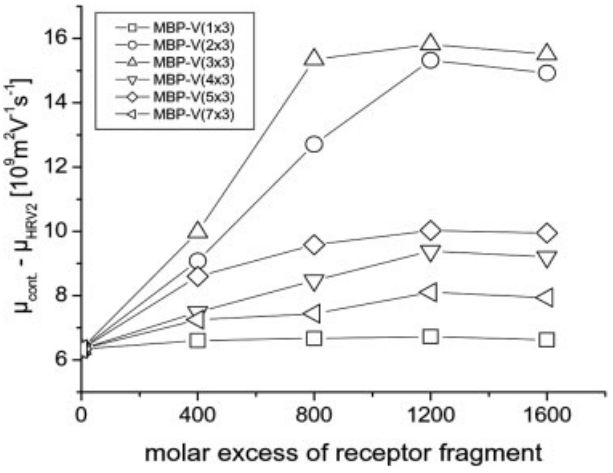

Figure 7. Shift in mobility (related to peak of internal standard) as function of molar excess of receptor over virus for different receptor fragments. Reactions were carried out as detailed in Fig. 6 with VLDL receptor concatemers with different numbers of ligand binding repeat number 3 (V3).

tions of virus and receptor. This indicates that the binding sites on the virus surface might be differently modified by the derivatization and/or that the specific binding activity of the ligands might vary because of incorrect folding of single receptors modules. Therefore, at this time, we did not attempt to derive quantitative data such as binding constants from these data. Nevertheless, the increase in the fraction of complexes formed with the number of concatenated modules reflects very well the data obtained *via* surface plasmon resonance and *via* FL correlation spectroscopy [26, 33].

It is not self-evident that the labeled virus still reacts with the receptors in spite of the most exposed lysines (that are involved in receptor attachment) being chemically modified by the FL dye. However, this is in agreement with our previous findings by using other dyes, and with the fact that the infectivity of the virus still remains upon labeling. The modification of the virus surface by the dye certainly affects receptor attachment, as can be concluded from the excess of receptor needed to give a noticeable complexation which is orders of magnitude larger as compared to experiments using native virus [31]. To avoid such restrictions, we intend to label the genomic RNA, on the one hand, and to label the receptor at the single N-terminal free amino group that arises upon enzymatic removal of the MBP. In this way, the interaction sites of the reactants remain intact allowing for the unperturbed determination of the physicochemical parameters of the virus-receptor interaction. This is one of the topics of our current investigations.

4 Concluding remarks

Intact rhinovirus particles were analyzed by microchip electrophoresis after staining of the viral capsid with Cy5 for LIF detection. This dye has an excitation wavelength (λ_{ex} is 649 nm) that is well-suited for the red laser of the employed commercial instrument, the Agilent 2100 Bioanalyzer. Compared to conventional CE in fused-silica capillaries, the analysis time with the microdevice was at least by one order of magnitude lower than with conventional CE. Separation from a contaminant, which also becomes labeled upon reaction of the virus sample with reactive Cy5, and from the excess free dye was improved by addition of SDS and varying its concentration in the BGE. Chip electrophoresis with the finally selected BGE (100 mM borate buffer, pH 8.3, containing 3.1 mM SDS), enabled an extremely rapid assessment of virus purity, and the investigation of bioaffinity reactions of labeled virus with a number of soluble artificial receptor fragments. This is exemplified by the investigation of the interaction between FL-labeled HRV2 and VLDL receptor derivatives with different numbers of ligand binding repeats. However, it is of note that FL labeling modifies the receptor binding sites on the viral capsid. Therefore, the affinity for the receptors of FL-labeled virions differs from that of the native viruses. To overcome this problem we are currently assessing the possibility to detect virus rendered fluorescent *via* incorporation of dyes into its RNA genome. This will allow for the determination of the binding parameters of the native partners using minute amounts of sample in short times on an electrophoresis microchip device.

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