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Labeling of Capsid Proteins and Genomic RNA of Human Rhinovirus with Two Different Fluorescent Dyes for Selective Detection by Capillary Electrophoresis

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During uncoating of human rhinoviruses, the innermost capsid protein VP4 and the genomic RNA are released from the viral protein shell. This process gives rise to subviral particles that are composed of the remaining three capsid proteins VP1, VP2, and VP3. The process is believed to take place in a sequential manner in that first VP4 is expelled resulting in A-particles sedimenting at 135S followed by the RNA resulting in B-particles sedimenting at 80S. Aiming at ultimately analyzing this process in vivo, we introduced two different fluorophores into the RNA and the viral capsid proteins, respectively. Incubation of the virus with RiboGreen resulted in formation of a RNA−dye complex with $\lambda_{ex}/\lambda_{em}$ = 500/525 nm, whereas subsequent derivatization of the viral protein shell in the same sample with AMCA-S introduced a label with $\lambda_{ex}/\lambda_{em}$ = 345−350/440−460 nm. In this way, both viral components could be selectively detected via fluorescence in a capillary electrophoresis system. The intact virus delivers two superimposed signals in the electropherogram. Derivatization of the free amino groups of the capsid proteins partially preserved the bioaffinity of the virus toward a synthetic receptor fragment, an artificial recombinant concatemer of repeat number 3 of the very low density lipoprotein receptor. Between 10 and 20% of the infectivity were recovered after labeling when compared to native virus. In addition to analysis of factors influencing the stability of the virus by CE, double-labeled virions might be useful for the investigation of the uncoating process by real-time confocal fluorescence microscopy.

Human rhinoviruses (HRVs) are icosahedral particles of 30 nm in diameter composed of four capsid proteins (VP1−VP4) that encase a genomic, positive strand (messenger sense) RNA of ~7100 nucleotides in length.[1] The 99 serotypes are classified into genus A and B based on phylogeny[2,3] or into a minor and a major group according to receptor binding.[4] Ten minor group HRV serotypes bind members of the low-density lipoprotein receptor (LDLR) family,[5] and 89 major group HRVs bind intercellular adhesion molecule 1 (ICAM-1).[6] Upon receptor-mediated endocytosis into the host cell, the RNA is released into the cytosol where it is translated into a polyprotein that is autocatalytically cleaved into capsid proteins and nonstructural (replicative) proteins. HRVs are a main cause of common cold infections and the reason for an enormous economic burden due to more than 1 billion cases annually in the United States alone.[7]

Aiming at investigating simultaneously both the viral protein components and the RNA during the endocytic pathway,[8] we attempted to label the capsid and the RNA separately in the same virion with two fluorescence (FL) dyes with different spectroscopic properties. This is based on our previous demonstration of labeling the genomic RNA of HRV2 with RiboGreen.[9] This dye penetrates into the intact virus shell and binds noncovalently to the RNA, resulting in an enormous increase of its intrinsic fluorescence.[10] We also demonstrated labeling of the viral capsid with fluorescein isothiocyanate (FITC),[11] a compound reacting with primary amines. We now report on the combined derivatization with RiboGreen (staining RNA) and with sulfosuccinimidyl-7-amino-4-methylcoumarin-3-acetic acid (AMCA-S; staining the capsid). These compounds are suitable for simultaneous detection due to

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their spectra: whereas AMCA-S is excited at 345−350 nm and emits at 440−460 nm, RiboGreen is excited at 500 nm and emits at 525 nm. The goal of our present work was to investigate the following: whether (i) the viral capsid can be reacted with AMCA-S resulting in an intact, fluorescing virion; (ii) it is possible to label the genomic RNA and the capsid specifically with the two different stains; (iii) bioaffinity is preserved after capsid derivatization and the virus still binds soluble receptor fragments; and (iv), the double-labeled virions are still infective.

EXPERIMENTAL SECTION

Instrumentation. The CE measurements were performed on an automated HP3D instrument (Agilent, Waldbronn, Germany) equipped with a diode array UV−visible detector. The fluorescence signal was monitored with a broadband fluorescence detector (Argos 250B, Flux Instruments, Basle, Switzerland), which was coupled with the CE instrument. Data acquisition and analysis of the UV and FL signals were performed using Agilent ChemStation Plus software. Untreated fused-silica capillaries were purchased from Composite Metal Services Ltd. with 75-μm i.d.; total length was 68.0 cm and effective length 59.5 cm for UV detection and 50.0 cm for FL detection, respectively. The capillary was packed in a standard Agilent cassette thermostated at 20 °C during all experiments.

Sample injection was accomplished by applying a pressure of 25 mbar to the inlet vial for 9 s. Positive polarity mode with 25 kV (368 V/cm) was used in all experiments with the detector placed at the cathodic side of the capillary. UV absorption was monitored at 205 and 260 nm. The excitation wavelength for fluorescence detection was selected with a broadband filter (240−400 nm) or a monochromator (350 and 500 nm); emission was measured at wavelengths selected using cutoff filters (435, 495 and 520 nm). Washing and conditioning of the capillary was routinely done by flushing with 1 mol/L sodium hydroxide solution for 1 min, followed by rinsing with water for 1 min and BGE for 2 min prior to each run.

Reagents. HRV2 was grown in HeLa-H1 cells (Flow Laboratories) in suspension culture and purified as described.[12] The viral protein concentration was determined spectrophotometrically from $A_{260} = 7.7$ for a 1 mg/mL solution.[13] Virus purity was checked by CE from the virus peak and corrected as described.[14] The concentration of the virus stock solution used was 3.5 mg/mL (0.40 μmol/L) in 50 mmol/L HEPES buffer (pH 7.5). Very low density lipoprotein receptor (VLDLR) module concatemers MBP-V33333 were constructed as described[15,16] and are a fusion of maltose binding protein (MBP), five copies of module 3 of VLDLR arranged in tandem, and a hexahistidine tag. The concentration of the receptor stock solution was 200 μg/mL (2.96 μmol/L). RiboGreen (in DMSO) was purchased from Molecular Probes (Eugene, OR) as part of a kit for quantifying RNA in solution. AMCA-S was from Pierce (Rockford, IL). FITC isomer I, sodium hydroxide, and sodium dodecyl sulfate (SDS) were from Sigma (Milwaukee, WI). Sephadex G-100 used to separate labeled virus from excess of fluorescence dyes and byproducts by size exclusion chromatography (SEC) was obtained from Amersham Bioscience (Uppsala, Sweden). The beads were swollen in 50 mmol/L borate buffer (pH 8.3) as described in the instructions. All other chemicals were purchased from E. Merck (Darmstadt, Germany).

The following buffers were used: (i) Virus separation was accomplished in 100 mmol/L boric acid containing 10 mmol/L SDS, adjusted to pH 8.3 with NaOH. (ii) Fluorescence labeling of the viral capsid and penetration of the capsid with RiboGreen were performed in 50 mmol/L borate buffer, pH 8.3. (iii) Elution of the labeled virus from the SEC columns was done in 50 or 25 mmol/L borate buffer, pH 8.3. All buffers were prepared in double-distilled water. Buffers were degassed by ultrasonication for 5 min and centrifuged for 5 min in a tabletop centrifuge from Eppendorf (model 5415D, Hamburg, Germany) at 11800*g* prior to CE analysis.

Procedures. For staining the RNA inside the viral capsid, the virus preparation was diluted 1:5 with labeling buffer, and 9.5 μL of this solution was incubated with 0.5 μL of RiboGreen (in DMSO) at 37 °C for 4 h, protected from light. For staining the viral capsid with AMCA-S, a mixture of 6 μL of labeling buffer, 2 μL of virus preparation, and 2 μL of AMCA-S of various concentrations (0.3, 1.0, 2.0, and 3.0 mg/mL) was incubated at 23 °C for 19 h in the dark. The respective molar excess of AMCA-S in the reaction solutions was 1590, 5340, 10 680, and 15 900. Excess dye was removed by SEC on self-packed Sephadex G-100 columns (1.0-mL bed volume, 3.8-cm bed height) equilibrated and eluted with 50 mmol/L borate buffer, pH 8.3. Fractions with a volume of 40 μL each were collected, and 8-μL aliquots were mixed with 2 μL of SDS (50 mmol/L) prior to CE analysis.[17] For the determination of the net mobilities, 0.2 μL of DMSO solution (diluted 1:100 in water) was added as EOF marker.

For staining both, the RNA and the capsid of the intact virus, the two labeling procedures described above were accomplished consecutively. After penetration of the capsid with RiboGreen, 2 μL of AMCA-S (2.2 mg/mL) was added for staining the viral capsid. Excess RiboGreen and AMCA-S were removed by SEC as described above.

To release the genomic viral RNA from the capsid, 8 μL of AMCA-S-labeled virus (main fraction from SEC) was mixed with 2 μL of 50 mmol/L SDS and incubated at 56 °C for 1 min by immersion in a water bath. For virus−receptor complex formation, MBP-V33333 and HRV2, labeled with AMCA-S, 4 μL of receptor stock solution (diluted 1:24 with 50 mmol/L borate buffer, pH 8.3) and 4 μL of virus from the main fraction of SEC (containing ~30% of the loaded virus) were mixed, resulting in a molar ratio of receptor to virus of 20:1. After 3 min, 2 μL of SDS (50 mmol/L) was added prior to CE analysis.

Infectivity Assay. Virus titer was determined as $TCID_{50}$ (tissue culture infectious dose that lyses 50% of the cells).[18] Briefly, HeLa-H1 cells were grown in minimal essential medium (MEM) containing 10% fetal calf serum (Life Technologies) supplemented

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with penicillin and streptomycin in 96-well tissue culture plates until ~60% confluent. The cells were then challenged with 2-fold serial dilutions of the virus samples in MEM containing 30 mmol/L $MgCl_2$ and 2% fetal calf serum. After incubation at 34 °C for 5 days, the plate was washed with water and stained with Crystal violet, and colorless wells were scored positive for virus infection. Wells appearing stained as a result of lack of infection were scored negative. Calculation of $TCID_{50}$ was carried out as described.[18]

RESULTS AND DISCUSSION

Labeling of the Viral Capsid with AMCA-S. AMCA-S was reacted with the virus using the conditions recommended by the supplier for protein derivatization (reaction in 50 mM borate buffer, pH 8.5). AMCA-S results in formation of amido groups with exposed lysines and with free N-termini of the proteins. This is in contrast to FITC, which gives rise to thiocarbamoyl derivatives. In our previous work,[11] we found that VP1 and also VP2 were modified with FITC, whereas VP3 and VP4 (located in the innermost part of the shell) were not labeled.

To find out the conditions for AMCA-S labeling resulting in the highest fluorescence signal at the given constant virus concentration of 70 nM (following from the virus stock concentration after adding the reagent's solution), the molar ratio of dye to virus was varied between 0 and 16 000. After completion of the reaction, the mixture was analyzed by CE. A typical electropherogram obtained of the reaction mixture without any purification step is shown in Figure 1. A number of peaks were detected (trace A), with the largest one corresponding to the excess of the reagent (compare with reagent blank, trace B) precluding unambiguous identification of the virus peak. When size exclusion chromatography was carried out prior to CE, the virus could be separated from the low molecular mass compounds (cutoff 100 kDa); the corresponding electropherogram is shown in trace C. The major peak, recorded at ~5.8 min, exhibits roughly the same electrophoretic mobility as seen earlier for native HRV2.[19] A second, smaller peak can also be found in the electropherogram (at 4.6 min, Figure 1 C); it presumably corresponds to the high molecular mass contaminant noted in our previous paper.[11] It is neither a polynucleotide nor a protein, but reacts with amine-reactive dyes; most likely it is not a constituent of the plasma membrane of the cells in which the virus is produced following infection since it was not observed upon carrying out exactly the same procedures with material recovered from mock-infected cells (data not shown).

It should be mentioned that we carried out SEC for several reasons: (i) The alternative to separate the labeled virus from the excess of reactant and the byproducts by modifying the CE conditions is severely restricted. It was found that only a small range of pH (and the presence of SDS) leads to reproducible electrophoretic results. (ii) The excess of the dyes has to be removed in the sample, otherwise it would probably impair the affinity reaction with receptors.

The net mobility, corrected for the electroosmotic flow, of the virus peak changed upon increasing the concentration of AMCA-S for derivatization. It slightly increased with increasing molar ratio between the dye and the virus and decreased then at ratios exceeding 1:6000 (see Figure 2). This effect cannot be explained by the chemistry of derivatization because the increase in mass is marginal, and the amino group of the lysine is substituted by a group that also contains a primary amine. A similar change of the mobility was also observed in our previous work with FITC, where we tentatively explained this effect by the complex migration mechanism in the CE system due to the presence of SDS micelles. At high molar ratios between the reactive dye and the virus, its mobility approached that of the high molecular mass contaminant, resulting in a deterioration of the resolution of contaminant and virus peak. We observed that derivatization with large molar ratios leads to a decrease in the peak area of the labeled virus; see Figure 2. The virus peak area increases first, reaches a maximum at a ratio between about 5000 and 6000, and then decreases further with increasing excess of dye. This behavior is not unusual for protein staining, as increasing the concentrations of fluorophor locally can result in quenching.[20,21] From these findings we deduced that a 6000-fold molar excess of AMCA-S over virus appears most favorable for efficient labeling. An electropherogram obtained under these conditions and recorded after removal of the low molecular mass constituents by SEC is shown in Figure 1, trace C.

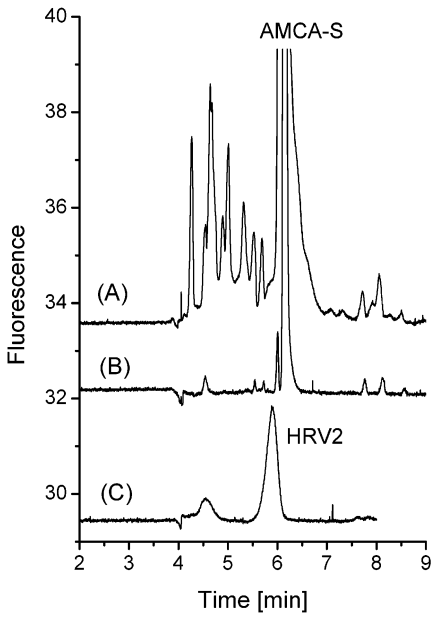

Figure 1. Electropherograms of reaction products of HRV2 after labeling the viral capsid with AMCA-S; detection by fluorescence ($\lambda_{ex}/\lambda_{em}$ = 240−400/435). (A) CE after incubation of the virus with AMCA-S at 23 °C for 19 h protected from light. The molar ratio of virus to reactive dye was 1:5340. One microliter of the reaction solution was diluted 1:20 with BGE prior to CE analysis. (B) Reagent blank. Concentration of AMCA-S was $^1/_5$ of that in (A). (C) CE of the labeled virus after SEC purification. Ten microliters of reaction solution was applied to a Sephadex G-100 column of 1.0-mL bed volume and eluted with 50 mmol/L borate buffer, pH 8.3; fractions of 40 μL each were collected. An aliquot of 8 μL from the fraction containing the majority of the virus was mixed with 2 μL of 50 mmol/L SDS. Separation conditions: uncoated fused-silica capillary (lengths 68.0 cm/50 cm, 75-μm i.d.); temperature, 20.0 °C; separation voltage, +25 kV; separation buffer, 100 mmol/L sodium borate, pH 8.3, 10 mmol/L SDS; injection at 25 mbar for 9 s.

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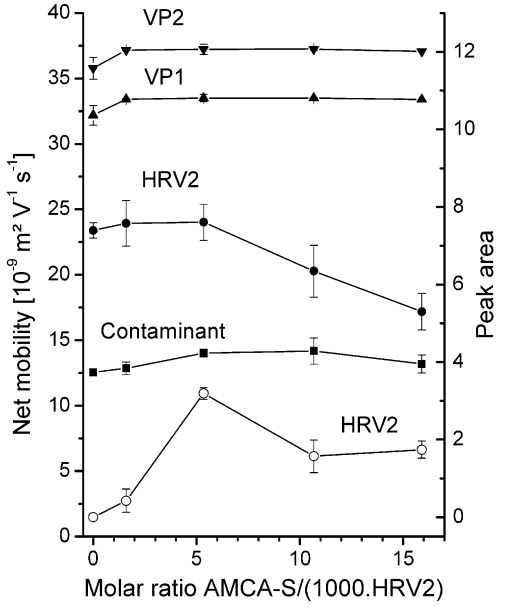

Figure 2. Net mobility of labeled virus, viral proteins, and contaminant and virus peak area as a function of the molar ratio between AMCA-S and virus. Full symbols, net mobilities (left axis); open symbols, peak area (right axis). Data are from three independent series of individual setups. Bars: standard deviation of measurement. Peak areas are in arbitrary units.

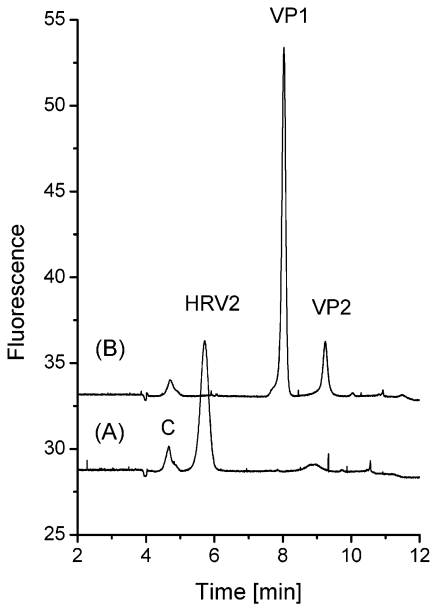

Figure 3. Electropherogram of AMCA-S-labeled HRV2 with fluorescence detection ($\lambda_{ex}/\lambda_{em}$ = 240−400/435). (A) Prior to and (B) after heat denaturation at 56 °C for 1 min. Two microliters of 50 mmol/L SDS was added to 8 μL of the main virus fraction recovered from SEC prior to injection. FL detection: excitation wavelength 240−400 nm (broadband filter); emission wavelength 435 nm (cutoff filter). Other experimental conditions as in Figure 1. C, Contaminant; VP1 and VP2, viral proteins.

To ensure the identity of the main peak with HRV2, additional experiments were carried out. Denaturation of the virion by exposure to 56 °C leading to the formation of subviral particles and interaction of the virus with receptor fragments have been previously used for this purpose. However, virus with a chemically modified surface might have lost its affinity for the receptors since lysine 224 in the HI loop of VP1 is known to be crucial for receptor interaction.[22]

In Vitro Uncoating. Exposure of HRV2 to temperatures of >50 °C leads to externalization of the innermost viral protein VP4, to the release of the genomic RNA, and, in the presence of SDS, to the decomposition of the capsid into the viral proteins VP1, VP2, and VP3 (SDS prevents loss of viral proteins during thermal exposition). These three proteins have molecular masses of 32 890, 28 982, and 26 088 Da, respectively (VP4 has 7350 Da). Out of the 289, 261, and 237 amino acids residues, respectively, 9, 8, and 6 are lysines. Note that 60 copies of each of these proteins compose the capsid and that VP1 contains the most exposed lysines; this agrees well with the observation that it is most strongly labeled with FITC.[11] From this it follows that the largest fluorescence peak in CE after disintegration of the virus should be VP1, followed by VP2 and VP3. VP4 does not give a signal, because it is internal.[23] The electropherogram obtained after denaturation of the AMCA-S-labeled virus by heat treatment (in the presence of 10 mM SDS) shows the disappearance of the peak assumed to be HRV2, with the concomitant appearance of two fluorescing peaks with higher net mobility (note that all analytes migrate against the EOF) (compare Figure 3B with A). These two peaks are similar to those obtained after FITC labeling, where they were identified as proteins by digestion with protease. The two peaks are most probably VP1 and VP2. In accord with FITC derivatization, the lysines of VP3 are seemingly not accessible for the reactive dye. Note that the sum of the peak areas (corrected for their different migration velocities) of VP1 and VP2 (Figure 3B) is three times higher than the peak area of the virus (Figure 3A). This is most likely caused by quenching of the fluorescence signal when the proteins are packed in the viral capsid.

Interaction with Receptor Fragment. Minor group HRVs, such as HRV2, bind VLDLR and other members of the LDLR family.[5] We have demonstrated high-resolution separation by CE of the complexes between HRV2 and artificial recombinant concatemers of repeat number 3 of VLDLR. From this we could convincingly derive that, at saturation, 12 receptor molecules attach per virion.[16] This is in agreement with theoretical predictions based on the capsid architecture and the structure and size of the receptor fragments. Here, we applied the reaction between HRV2 and the receptor concatemer to confirm the identity of the virus peak by incubating AMCA-S-labeled HRV2 with a 20-fold molar excess of MBP-V33333. The resulting electropherogram is shown in Figure 4. The large peak (trace A) with the mobility of HRV2 decreased with the concomitant appearance of six new peaks (trace B). Interestingly, a very similar picture was seen for the reaction of the native virus with this receptor fragment. In that case, the peaks could be related to the complexes of the virus with 1, 2, 3, etc., receptor fragments. From the results shown in Figure 4, there is clear evidence that the virus, although possessing a chemically modified surface, is still able to bind its receptors. These results confirm that the peak stems from the virus.

Combined Labeling of Viral Capsid and RNA. To select the appropriate settings for fluorescence detection, we checked the sensitivity and selectivity using solutions of the pure dyes.

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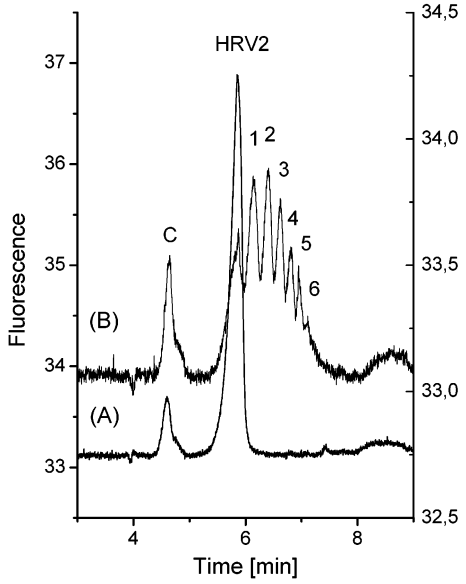

Figure 4. Virus with AMCA-S-labeled capsid binds recombinant receptor fragment MBP-V33333. (A) Eight microliters of labeled virus (main fraction eluted from SEC) plus 2 μL of 50 mmol/L SDS (control). (B) Four microliters of main fraction from SEC was incubated with 4 μL of receptor stock solution (diluted 1:24 with 50 mmol/L borate buffer, pH 8.3) for 3 min at 23 °C, and then 2 μL of SDS (50 mmol/L) was added before injection. HRV2 concentration is 6.2 nmol/L, the molar ratio of virus to receptor is 1:20. Peaks are numbered to indicate the stoichiometry between virus and receptor. Left axis, trace (A); right axis, trace (B). Separation conditions were as in Figure 1. C, contaminant.

Table 1. Detection Selectivity of RiboGreen and AMCA-S[a]

$\lambda_{ex}/\lambda_{em}$ (nm)	500[b]/520[c]	350[b]/435[c]
RiboGreen	0.40	0.008[d]
AMCA-S	0.028[d]	0.41

[a] Heights (in mV) of the electrophoretic peak of RiboGreen ($\lambda_{Ex}/\lambda_{Em}$ = 500/525) and AMCA-S ($\lambda_{Ex}/\lambda_{Em}$ = 345−350/440−460) at different excitation and emission wavelengths. Electrophoretic conditions as in Figure 1. Concentrations of injected solutions of AMCA-S 0.23 μmol/L, of RiboGreen ~20 μmol/L (20-fold diluted stock solution). [b] Monochromator. [c] Cutoff filter. [d] Standard deviation of baseline noise.

With the instrumentation available, two possibilities exist to select the excitation wavelength: by the aid of a monochromator or by a band-pass filter. The maximum absorbance of RiboGreen is at 500 nm and that for AMCA-S is at 345−350 nm. The emission wavelengths are 525 (RiboGreen) and 440−460 nm (AMCA-S). From Table 1 it can be seen that the use of the monochromator enables the selective detection of the dyes as indicated by the electrophoretic peak heights obtained from plain dye solutions. Recording the FL signal of both dyes at 500/520 nm, only baseline noise was observed for the AMCA-S trace. This noise (expressed by its standard deviation) is less than 7% of the peak height of RiboGreen injected at 20 μmol/L. By analogy, AMCA-S displays a peak at 350/435 nm and only baseline noise at 500/520 nm. In case a band-pass filter was used for excitation, the sensitivity for RiboGreen was three times higher (because the band-pass filter allows a much higher light intensity to pass) for AMCA-S the increase was 28-fold. Note that under this condition the excitation wavelength is much below the absorption maximum for

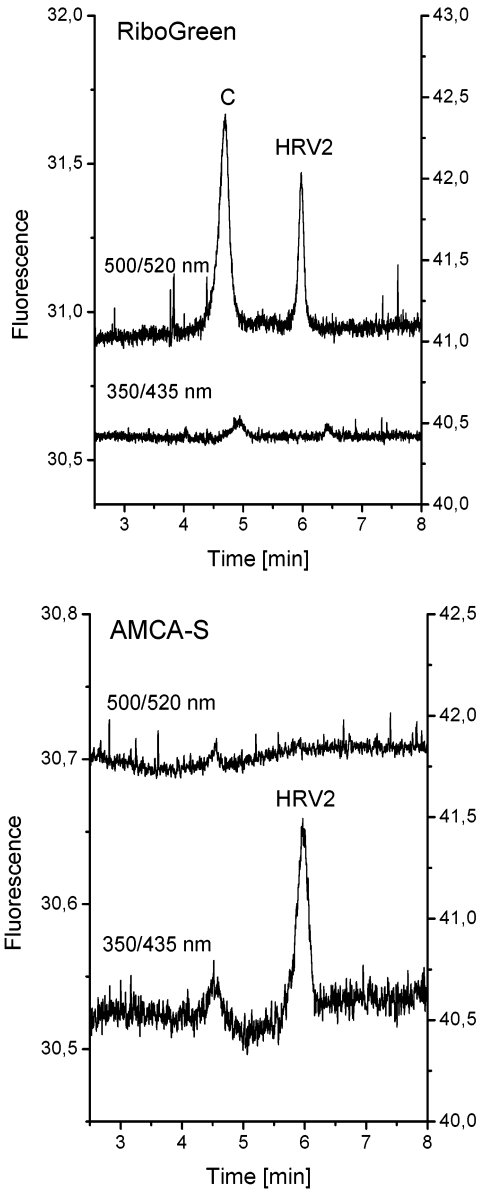

Figure 5. Electropherograms of labeled HRV2 with fluorescence detection selective for viral RNA or for the capsid. Top: CE of the RNA-labeled virus after penetration with RiboGreen. Bottom: CE after capsid labeling with AMCA-S. Experimental conditions: FL detector with monochromator (λ_{ex} = 500 or 350 nm) and cutoff filter (λ_{em} = 520 or 435 nm). Left axis, record at 350/435 nm; right axis, 500/520 nm. Experimental conditions as in Figure 1. C, contaminant.

RiboGreen. For AMCA-S, this effect is even more pronounced, because the absorption maximum lays within the filter range. The higher sensitivity is at the expense of detection selectivity; therefore, we used the more selective conditions.

The electropherograms of virus stained either with RiboGreen or with AMCA-S are shown in Figure 5. RiboGreen labeling was recorded at 500/520 nm (Figure 5, top); at this wavelength, the contaminant was also revealed. At the setting for detection of AMCA-S ($\lambda_{ex}/\lambda_{em}$ = 350/435 nm), only a tiny peak is seen at the position of the virus; seemingly, this can be attributed to autofluorescence of tryptophan and tyrosine contained in the capsid proteins. The corresponding area is only 1% of the peak monitored at 500/520 nm. This small peak is observed also for unmodified virus (data not shown); hence it has nothing to do with RiboGreen.

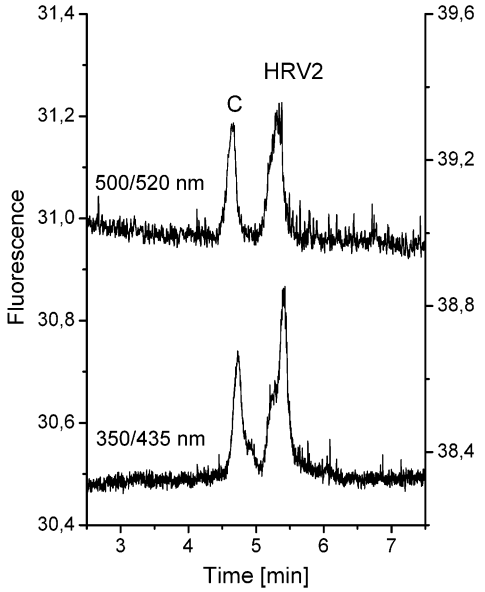

Figure 6. Electropherogram after penetration of the virus with RiboGreen and subsequent capsid labeling with AMCA-S recorded at the respective wavelengths (RiboGreen, 500/520 and AMCA-S, 350/435). Left axis, record at 350/435 nm; right axis, 500/520 nm. Separation conditions as in Figure 1. C, contaminant.

The electropherograms monitored by selective detection of AMCA-S ($\lambda_{ex}/\lambda_{em}$ = 350/435) are shown in Figure 5, bottom. There is no peak at the alternative wavelengths of 500/520 nm. It can be seen from both figures that the peaks, although being far from the detection limit, are somewhat noisy. This is due to the limited detection sensitivity of the detector used, compared, for example, to the LIF detector, necessitating a high signal attenuation. However, the commonly used argon ion laser has an excitation wavelength of 488 nm and is, therefore, not well-suited to AMCA-S. The sensitivity might be increased by operating with a broadband excitation filter instead of the monochromator. Indeed, the sensitivity increased by a factor of 28, but at the same time the detection selectivity was lost as pointed out above.

For the combined staining of both capsid and RNA in the same virus sample, the virus was first incubated with RiboGreen to form the RNA complex, and then it was reacted with AMCA-S that was added without separating the virus from excess RiboGreen. We verified by CE of a mixture of the pure dyes that they do not interfere with each other (data not shown). Electropherograms obtained after the reactions and SEC purification are shown in Figure 6. Both the capsid−AMCA-S and the RNA−RiboGreen derivatives can be detected at their specific wavelengths, without mutual interference. Slight changes of the migration times are caused by small variations of the electroosmotic flow velocity.

We observed previously that a number of factors inhibit the penetration of RiboGreen into the capsid to various extents. For example, binding of isoxazol derivatives that are antivirally active, into the hydrophobic pocket of the virion,[24] results in a diminution of complex formation. Ca^{2+} ions or cysteine and cysteamine were found to impede penetration (not shown). Therefore, to exclude that derivatization of amino acid residues at the capsid surface by reaction with AMCA-S modifies the kinetics and extent of penetration, we also carried out the incubation with RiboGreen after the AMCA-S reaction. The resulting electropherograms were indistinguishable (not shown).

(24) McKinlay, M. A.; Pevear, D. C.; Rossmann, M. G. *Annu. Rev. Microbiol.* **1992**, *46*, 635−654.

Table 2. Infectivity of Dual-Labeled Virus[a]

sample	$TCID_{50}$/mL
fraction 12	6.2×10^8
fraction 13	2.4×10^8
HRV2 reference (60-fold dilution)	3.6×10^9
HRV2 stock solution	1.9×10^{11}

[a] Virus was incubated with RiboGreen at 37 C for 4 h in the dark, followed by reaction with AMCA-S, and separated from excess low molecular weight material by SEC on Sephadex G100 in borate buffer, pH 8.3. Fractions, 40 μL each, were collected. From two fractions with ~33 (12) and ~29% (13) of the total virus recovered, the infectivity was determined. The virus concentration in the individual fractions was determined by CE. Fractions and reference sample correspond to a 60-fold dilution of the initial virus preparation. $TCID_{50}$/mL, tissue culture infectivity dose that infects 50% of the cells.

Infectivity of Double-Labeled Virus. For infection, the virus attaches to a specific receptor at the cell surface. Subsequent entry takes place by clathrin-mediated endocytosis via early and late endosomes, where the release of the RNA into the cytosol takes place. Surface derivatization of the virion might prevent cell attachment and complex formation between the dye and the genomic RNA might hamper infection. Therefore, we compared the infectivity prior and after derivatization. As summarized in Table 2, the $TCID_{50}$ of the virus preparation was 1.9×10^{11}/mL. The fractions of the double-stained virus collected from SEC had virus concentrations of ~$^1/_{60}$ of this initial virus stock. As a reference, a virus sample was subjected to the same conditions (incubation at 37 °C for 4 h followed by exposure to 23 °C for 19 h) as used for staining, but without adding dyes, as the infectivity might be lost just by the incubation. The sample was finally diluted to the same concentration as the labeled virus had after SEC. Determination of the $TCID_{50}$ revealed a value of 3.6×10^9/mL, which is lower by a factor of 53 as compared to the original virus sample, in approximate agreement with the 60-fold dilution. The two main fractions of the derivatized virus samples collected after SEC have $TCID_{50}$ values of 6.2×10^8 and 2.4×10^8/mL, respectively. This is about 5−10 times lower than the untreated virus; i.e., on average, 10−20% of the infectivity is preserved even after chemical modification. This result is in agreement with that of derivatization of the virus capsid by FITC. The remaining infectivity (which qualitatively agrees with the preserved bioaffinity of the stained virus toward receptor fragments) is most probably caused by the incomplete reaction of the capsid lysines with the fluorescence reagent. There are still sufficient lysines available for recognition of receptor sites. The amino acid residues in VP1 modified by AMCA-S might be identified by mass spectrometry. This is the topic of current research.

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