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Capillary isotachophoresis with fiber-optic Raman spectroscopic detection
Performance and application to ribonucleotides

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Abstract

A fiber-optic Raman probe fitted with a microscope objective was used to obtain on-line normal Raman spectra of adenosine 5′-monophosphate, cytidine 5′-monophosphate, guanosine 5′-monophosphate and uridine 5′-monophosphate separated by capillary isotachophoresis. With multimode optical fiber, the system interrograted a 40-μm length of capillary. Fiber-optic coupling facilitated use of an unmodified spectrograph and conventional capillary mounting systems. Raman spectra were excited with a 2W 532 nm $NdYVO_4$, laser as the excitation source, with collection of 1 spectrum per second. Even at $2 \cdot 10^{-5}$ *M* initial concentration, Raman spectra were obtained at a good signal-to-noise ratio. © 1998 Elsevier Science B.V.

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1. Introduction

Intra-capillary normal Raman spectroscopy is readily coupled to capillary zone electrophoresis [1] or capillary isotachophoresis [2–4]. Preconcentration of some form is needed because Raman spectroscopy has good mass detection limits, but poor concentration detection limits. cITP is the preferred mode. The Raman spectroscopy optical system can interrogate a short section of capillary efficiently, thus maximizing the resolving power of ITP.

In all previous work, we employed a Raman microprobe as the detection system. A microprobe consists of an epi-illumination microscope optically coupled to a spectrograph and detector [5]. 'Interfacing' a capillary to this instrument consists of little more than providing means to hold it on the microscope stage. Although there is no difficulty in so doing in a purpose-built electrophoresis system, conventional instruments must be modified so that about 20–40 cm of capillary is external to the safety enclosure. This requirement severely compromises many instruments, including most commercially available capillary electrophoresis systems.

Fiber-optic probes are now commonly employed in Raman spectroscopy where there is a need for remote analysis of samples [6]. Because laser radiation is conducted to and from the sample via optical fibers, Raman spectra can be obtained from samples as far as 50–100 m away. In situ Raman monitoring in hostile environments is now routine.

Of the various fiber-optic probe designs which

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have been proposed, the best performance is usually obtained with coaxial filtered probes [6]. In these probes, separate optical fibers are employed to deliver laser radiation to the sample and collect Raman scatter. The optics can be integrated into a compact probe head which can be fitted with an achromat or a microscope objective, depending on the needs of the experiment.

These probes are ostensibly the most versatile accessory in Raman spectroscopy. The only consideration for setting up the fiber optic Raman probe is the interfaces of the optical fibers to the excitation laser and spectrograph. To obtain the highest efficiency, the excitation laser should be launched into the fiber with a numerical aperture matched optic. Unmatched numerical apertures can result in reflection or refraction of the light into the cladding medium thus losing total internal reflection. Commercial fiber couplers are available that hold the focusing objective and fiber stationary and allow sub-micron resolution positioning.

On-capillary normal Raman spectroscopy requires analyte concentrations near 10^{-2}–10^{-3} M for good signal-to-noise ratios at short acquisition times. We use two on-capillary preconcentration techniques, field amplified injections and isotachophoresis. Field amplified injections occur when the sample solution is less concentrated than the buffer electrolyte in the capillary. The conductivity difference between the sample solution and buffer electrolyte produces a differential electric field. The enhancement is approximately the ratio of the buffer concentration to that of the sample solution concentration. We have experienced $10^{3}\times$ gain in sample injection with field amplified injections [1].

Isotachophoresis is often used in tandem with capillary zone electrophoresis for preconcentration [7,8]. Analytes are injected between a leading and trailing electrolyte solution and at a steady-state concentration to nearly the leading electrolyte concentration. Once steady state is achieved, the zones maintain their properties. The concentration, zone width and intermixing regions remain largely unchanged until the analytes elute from the capillary. When multiple analytes are separated, the concentration of each analyte zone is determined by that of the preceding zone. Therefore, the stacking efficiency of the analytes decreases from the higher mobility analytes to the lower mobility ones. Enhancement factors up to $10^{4}\times$ have been observed with field amplified injections used in combination with ITP of ultra dilute solutions [2,3].

In this communication, we present the acquisition of intra-capillary normal Raman spectra with a fiber-optic Raman probe. The performance and feasibility are examined by measurements of intra-capillary Raman spectra under static conditions. The fiber probe is used to monitor the cITP separations of adenosine 5′-monophosphate (AMP), cytidine 5′-monophosphate (CMP), guanosine 5′-monophosphate (GMP) and uridine 5′-monophosphate (UMP) by their Raman spectra.

2. Experimental

2.1. Instrumentation

The experimental setup used to collect the Raman scattering from the capillary is shown in Fig. 1. A solid-state $NdYVO_4$ laser (Spectra-Physics, Mountain View, CA, USA) which emits 2 W of 532 nm radiation is used as the excitation source. A Kaiser Optical Systems (Ann Arbor, MI, USA) Mark II fiber-optical probe fitted with an Olympus 20X/0.46NA MS plan achromat (Olympus America, Lake Success, NY, USA) was used to interrogate the capillary.

The optical design of the fiber-optic probe is sketched in Fig. 2. Briefly, laser light is delivered though a 50-μm core optical fiber, filtered through a transmission grating and reflected from a holographic notch filter into a microscope objective. Raman scattered light is collected through the same microscope objective and filtered through two notch filters to remove back scattered laser light. The filtered Raman scattered light is carried to the Raman spectrograph through a 100-μm core optical fiber. Both the excitation and collection fibers were 5 meters long.

The Raman scatter from the collection fiber is delivered to an 85-mm focal length axial transmissive spectrograph (Kaiser Optical Systems, HoloSpec f/1.8i) [9] fitted with a holographic transmission grating and a cryogenically cooled CCD camera (CH-270, Photometrics, Tucson, AZ, USA). A 50-

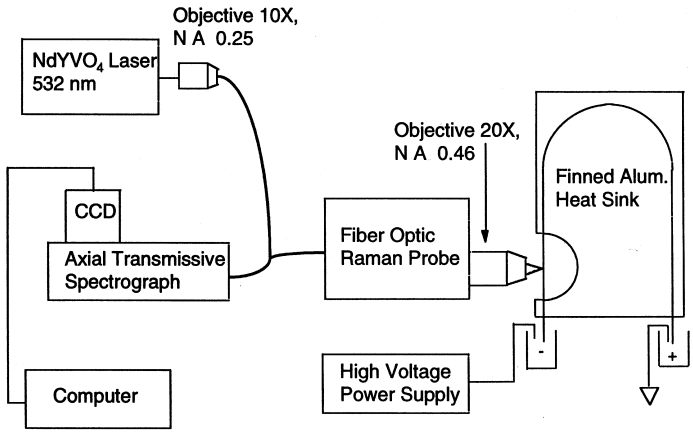

Fig. 1. Block diagram of the fiber-optic Raman probe system.

μm entrance slit provided resolution of about 8 cm^{-1}. Spectra were acquired using CCD 9000 software (Photometrics) and processed in GRAMS 386 (Galactic Industries, Salem, NH, USA) and Matlab 5.0 (The Math Works, Natick, MA, USA). Spectra were collected every 1.2 s, allowing 1-s integration and 200-m transfer and storage time.

The locally constructed isotachophoresis apparatus has been previously described [2]. Briefly, it consisted of a −30 kV high-voltage power supply (Model PS/LG-30R-5, Glassman High Voltage, Whitehouse Station, NJ, USA) and a Plexiglas safety enclosure for the high-voltage buffer reservoir. The electrophoresis capillary was mounted in an aluminum heat sink/radiator [10]. The radiator was convectively cooled with forced air. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were used with inner diameters ranging from 50 μm to 250 μm and an outer diameter of 365 μm. ITP was performed in 50 μm I.D. capillaries coated with 3% *T* linear polyarylamide [11] to minimize electroosmotic flow [*T*=(g of acrylamide+g of *N,N′*-methylenebisacrylamide)/100 ml of solution]. Samples were electrokinetically introduced at 200 V cm^{-1} and injection times ranged from 60 to 120 s. The distance from the capillary entrance to the detection window was 28 cm and the total length was 30 cm. Field strengths ranged from 66 V cm^{-1} to 400 V cm^{-1}, corresponding to applied voltages of 2 kV and 12 kV.

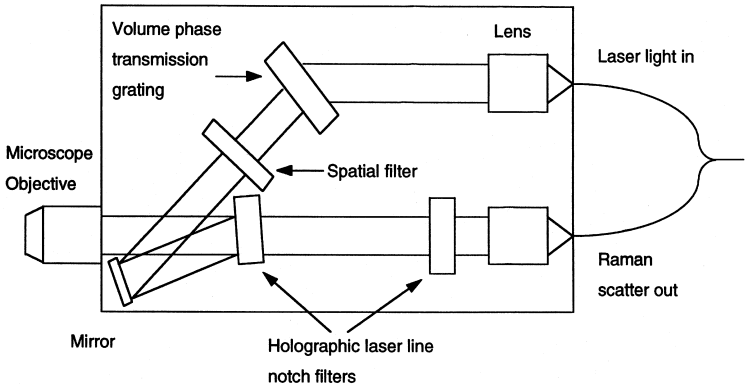

Fig. 2. Optical schematic of the Kaiser Optical Systems, Mark II fiber-optic Raman probe. Component details in Section 2.1.

2.2. Reagents

Sodium sulfate (Baker, Phillipsburg, NJ, USA) hexane, 4-morpholinopropanesulfonic acid (MOPS), AMP, GMP, UMP and CMP (Aldrich/Sigma, St. Louis, MO, USA) were used as received. All solutions were prepared with Type I deionized water.

3. Results and discussion

3.1. Optics of the fiber probe

The fiber-optic probe head is much smaller than a microscope. Unlike the Raman microprobe, we were able to use it inside a conventional capillary electrophoresis safety enclosure. Because the same microscope objective is used as the focusing and collection optic, the probe was easily focused into the electrophoresis capillary. However, the optical behavior of the fiber probe was not identical to that of a microscope.

We have compared the intensity of the Raman spectrum obtainable with the fiber optic Raman probe to that obtainable at equal laser power with the Raman microprobe used in our previous experiments [4]. For this test, we measured the peak intensity of the 987-cm^{-1} sulfate band of 0.1 *M* sodium sulfate in a 50-μm capillary under static conditions. The intensities were normalized for exposure time and delivered laser power. The normalized signal through the fiber-optic Raman probe was 10 counts per second mW, while the signal though the microprobe was 36 counts per second mW. The signal intensity from the fiber probe is only 28% of the intensity from a direct-coupled Raman microprobe.

Coupling losses into and out of the optical fibers of the Raman probe result in a lower intensity signal than is obtainable with the same laser power delivered to a microscope-based Raman microprobe. The measured transmission through the laser delivery fiber is 75%. The transmission through the collection fiber is 71%, presumable because it was less perfectly cleaved or polished than the input fiber. Insertion losses limit the maximum Raman signal intensity obtainable from this probe to 53% of the intensity obtainable through an otherwise identical direct-coupled system.

Fiber coupling losses alone cannot account for the decreased efficiency. The insertion losses in the internal filtering system of the fiber optic probe and similar to those of the filters and beam splitters used in the Raman microprobe. These elements do not account for the difference in efficiencies. As we show below, the multimode delivery fiber also is a major source of low optical throughput.

We measured the diameter of the focused output from the probe to be 40 μm and the length of the focal cylinder at the points where the diameter is twice the focused diameter (80 μm) to be 475 μm. With the same objective, the direct-coupled microprobe focused the laser beam to a cylinder of 6-μm diameter and 8-μm long. These measurements were made in air. Because the capillaries act as strong focusing lenses, the actual dimensions of the focal cylinders in the capillary will be smaller, but the multimode input fiber will still generate a focal cylinder whose greater than the capillary diameter.

To verify that a long focal cylinder is a major loss generator, we measured the intensity of the 889-cm^{-1} band of hexane as a function of capillary diameter. In Table 1 we summarize this data normalized to the signal from an infinitely thick (>1 cm deep) layer of hexane. Taking fiber insertion losses (T=53%) and capillary over-filling (T=47%, from Table 1) to be the important sources of low throughput, we expect that the fiber-optic probe should generate a signal which is the product of their insertion losses. The probe should generate a signal approximately 25% as large as obtained from a direct-coupled instrument. This calculated number is in good agreement with the measured value, 28%, in comparison with the direct-coupled Raman microprobe.

Our ITP-Raman spectroscopy experiments are performed in 50-μm I.D. capillaries. Small I.D.

Table 1
Effect of capillary internal diameter (I.D.) on Raman intensity of hexane 889-cm^{-1} band measured through a fiber-optic probe

Capillary I.D. (μm)	Fiber probe (relative intensity)
Bulk	1.0
200	0.93
100	0.86
75	0.69
50	0.47

capillary is used to maximize heat dissipation and allow use of high running voltages with high ionic strength buffers. As shown in Table 1, a capillary internal diameter of 250 μm or greater is needed to recover the maximum signal from the fiber optic probe. Less that half of the available signal is recovered from a 50-μm I.D. capillary. Even with a 75-μm I.D. capillary, which is the largest diameter generally recommended for electrophoresis, little more than 2/3 of the available signal is recovered.

There are two ways to improve this situation. A delivery fiber with smaller core diameter would generate a focal cylinder with smaller dimensions. Below 50 μm, the next available core diameter in silica fiber is 10 μm. Most 10-μm fibers are designed for illumination with no more than 100–200 mW. Although such a fiber would generate a focal cylinder whose dimensions are about 1/5 of those obtained with the more robust 50-μm fiber, it could not be used with the 1–2 W lasers necessary to generate intra-capillary Raman spectra at collections times of 1 s or less.

Alternatively, one could use an objective with a higher numerical aperture (NA), although higher NA is accompanied by smaller working distances. Suitable objectives are unavailable in the Olympus MS plan achromat range, although they may be available in other objective designs. Within the constraints of our equipment, a 20×/0.46NA objective is the best choice for use with the fiber-optic Raman probe.

Fig. 3 shows the fiber-optic probe Raman spectra of the ribonucleotides used in this work. The spectra were obtained with 1 s acquisition during ITP. The ribonucleotides have strong and characteristic bands in the 1200–1600 cm^{-1} region arising from the nucleic acid bases and ribose moieties, as well as characteristic nucleotide bands in the 700–1600 cm^{-1} region. In general, the spectra resemble those of related ribonucleotides which we have previously reported under ITP conditions [2,4].

Although the absolute magnitude of the signal is smaller than obtainable with a Raman microprobe, the quality of the spectra is not very different from those we have previously reported. The limiting noise is associated with subtraction of the water background, not with the absolute magnitude of the signal. The smaller signal generated with the fiber-optic probe is not a limitation of the technique.

3.2. ITP-raman spectroscopy with the fiber-optic probe

Fig. 4 shows the complete Raman isotachopherogram of AMP, CMP and GMP from a solution at a starting concentration of $3.3 \cdot 10^{-5}$ *M* in each ribonucleotide. Experimental conditions are summarized in the figure caption. The elution order is CMP, GMP and AMP. The field strength was held at 200 V cm^{-1} for approximately 7 min to allow zone formation and then reduces to 66 V cm^{-1} to allow adequate time for signal collection.

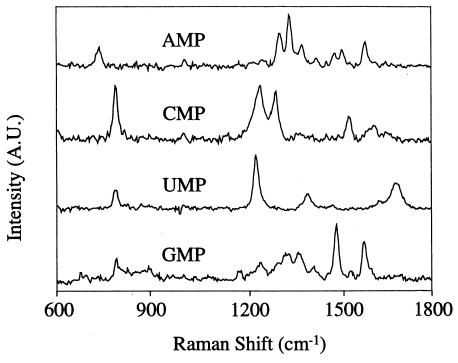

Fig. 3. Fiber-optic Raman spectra of AMP, CMP, UMP and GMP contained in a 50-μm I.D. capillary. The spectra were obtained during isotachophoresis, at 1-s integrations, 2W 532 nm excitation.

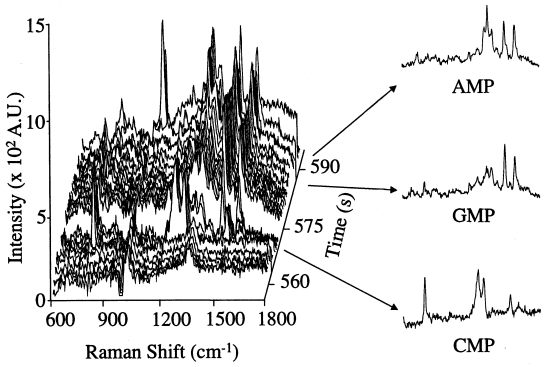

Fig. 4. On-capillary Raman isotachopherogram of AMP, CMP and GMP at a starting concentration of $3.3 \cdot 10^{-5}$ *M*. Leading electrolyte 0.1 *M* Na_2SO_4; trailing electrolyte 0.1 *M* MOPS. Sample introduction, 120 s at 200 V cm^{-1}; separation at 200 V cm^{-1} and 66 V cm^{-1}; 2 W 532 nm excitation.

The three ribonucleotide zones are easily identified by their Raman spectra, although each individual spectrum is slightly obscured in presentation of over 60 spectra in the stacked plot format. Individual frames from each analyte are displayed at the right of each isotachopherogram. The spectra are of adequate quality to allow positive identification. Even better signal-to-noise ratios can be obtained by averaging the spectra of individual species or, equivalently, by using factor analysis on the ensemble of spectra [3].

Fig. 5 shows the Raman isotachophemgram of AMP, CMP, and UMP from a solution at a starting concentration of $1.9 \cdot 10^{-5}$ *M*. The separation protocol is similar to that in the previous example. The sample was introduced at 183 V cm^{-1} (5.5 kV) for 60 s. The separation was run at 400 V cm^{-1} and maintained for 3 min before reduction to 66 V cm^{-1}. To eliminate bubble formation from dissolved gases, the sample and electrolyte solutions were actively degassed with helium for about 5 min before use.

The combination of low starting concentration and short introduction time produces short zones of concentrated analytes. At the 7-min run times, the short zones allow easy visualization of the diffusional intermixing regions between the pure analyte zones. These zones have been deliberately maximized to demonstrate signal recovery techniques. More realistically, even run times as short as a minute should produce interdiffusion widths longer than the 40-μm length interrogated by the fiber-optic Raman probe.

Even under these adverse conditions, we can recover the spectra of each analyte and construct the zone profiles of each, using factor analysis. In our implementation factors represent zone profiles and scores represent the Raman spectra of the analytes. The eigenvectors from which the factors are derived are rotated using the Varimax procedure [12] with respect to the time axis to produce zone profiles of physical significance. The scores require no rotation and do not contain artifacts which might conceivably result from constraints imposed on the eigenvectors rotation. In Fig. 6 the rotated eigenvectors, which represent interdiffusing analyte zones, are shown for the separation in Fig. 5. The nucleotides in the zone are unambiguously identified by their Raman spectra, which are identical to those shown in Figs. 3–5.

We have demonstrated that a fiber-optic Raman probe can be used to obtain real-time intra-capillary Raman spectra during ITP. The commercially available probe is less expensive and even easier to use in this application than a complete microscope-based Raman microprobe. Some performance improvement could be obtained with minor modifications to the optics of the system. Most importantly, the fiber probe is compatible with much of the world's inventory of commercial and purpose-built capillary electrophonetic instrumentation.

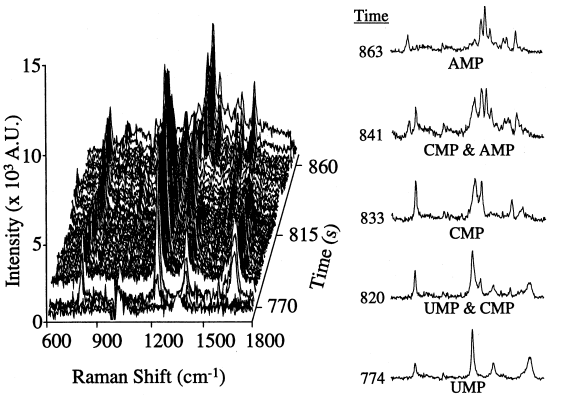

Fig. 5. On-capillary Raman isotachopherogram of AMP, CMP and UMP from a starting concentration of $1.9 \cdot 10^{-5}$ *M*. Sample introduction, 60 s at 183 V cm^{-1}; separation at 400 V cm^{-1} and 66 V cm^{-1}. Solutions degassed, forced air convective cooling. Other conditions as in Fig. 4.

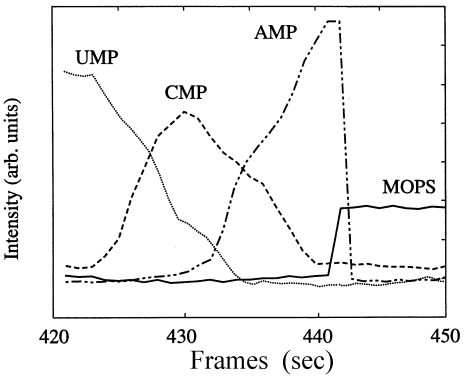

Fig. 6. Zone profiles of AMP, CMP and UMP and the trailing electrolyte, MOPS recovered by factor analysis of the data presented in Fig. 5.

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