Anal. Chem. **2002,** *74,* 4702−4708

Automated Affinity Capture−Release of Biotin-Containing Conjugates Using a Lab-on-Valve Apparatus Coupled to UV/Visible and Electrospray Ionization Mass Spectrometry

Yuko Ogata,[†] Louis Scampavia,[†] Jaromír Růžička,[†] C. Ronald Scott,[‡] Michael H. Gelb,*[,†,§] and František Tureček*[,†]

Departments of Chemistry, Pediatrics, and Biochemistry, University of Washington, Seattle, Washington, 98195-1700

We report a new method for automated affinity capture and release of biotin-containing conjugates on immobilized streptavidin using a lab-on-valve (LOV) bead injection apparatus. The apparatus is also coupled to UV/visible and electrospray ionization mass spectrometry (ESI-MS) for monitoring the captured and released biotin-containing conjugates. Dissociation rate constants for release from streptavidin of two chromophore-tagged biotin conjugates were measured by UV/visible spectrometry and the dissociation was simultaneously monitored by ESI-MS. The LOV-ESI-MS instrument was also used for repetitive assays of lysosomal β-galactosidase in human cell homogenates. Fast analysis in 4.5 min/full cycle and robust operation in 60 repetitive analyses are demonstrated that are promising for transfer of the LOV-ESI-MS technology into clinical practice.

The strong noncovalent interaction of biotin and biotinylated biomolecules with the proteins avidin[1] and streptavidin[2] forms the basis for highly selective affinity separations.[3−6] Separation is typically achieved by capturing biotin-containing conjugates with avidin or streptavidin that is immobilized on agarose or Sepharose beads and washing away nonspecifically bound impurities. However, due to the extremely strong affinity, retrieval of the sample after separation can be difficult and the release step may require specific conditions that allow the disruption of the noncovalent interaction without affecting the biotin-containing conjugates[6] or cleavage of the linker between biotin and the biotin-containing conjugates. To retrieve the samples effectively, several methods have been developed that include the use of monomeric avidin,[7] various forms of mutant streptavidin[8,9] such as temperature-sensitive streptavidin−polymer conjugates,[10] photocleavable biotin conjugates,[11,12] and pH-sensitive biotin conjugates such as iminobiotin.[13] For the above methods, dramatic change in pH or temperature, UV radiation, or excess biotin is needed for sample retrieval, which may in some cases damage the sample or require extra steps before the sample can be analyzed by mass spectrometry. In addition, due to the fact that the avidin/streptavidin beads need to be replaced typically after only a few uses, automation of the purification procedure has been difficult, and so far no such automated system has been directly coupled to electrospray ionization mass spectrometry (ESI-MS).

The nature of the noncovalent bonding was elucidated from the X-ray crystal structures of streptavidin and a streptavidin−biotin complex.[14,15] Streptavidin is a tetrameric protein in which the monomeric units are folded as eight-stranded antiparallel β sheets and held together by hydrogen bonds. Biotin fits in the pocket at the ends of the β sheet barrels, which contain mainly aromatic (Trp, Tyr) and polar amino acids (Asn, Ser, Thr). Upon binding, biotin displaces several molecules of water bound in the pocket and is oriented such that the bicyclic ring system points into the pocket and the carbonyl oxygen of the ureido group interacts by hydrogen bonding with Tyr-43, Asn-23, and Ser-27. The biotin carboxylate group points toward bulk solvent but is also stabilized by hydrogen bonding to Asn and Ser residues in the protein.

* Corresponding author. Tel: (206) 685-2041. Fax: (206) 685-3478. E-mail: turecek@chem.washington,edu.

[†] Department of Chemistry.

[‡] Department if Pediatrics.

[§] Department of Biochemistry.

(1) Green, N. M. *Adv. Protein Chem.* **1975**, *29*, 85−133.

(2) Stapley, E. O.; Mata, J. M.; Miller, I. M.; Demny, T. C.; Woodruff, H. B. *Antimicrob. Agents Chemother.* **1963**, *3*, 20.

(3) Green, N. M. In *Methods in Enzymology*; Wilchek, M., Bayer, E. A., Eds.; Academic Press: San Diego, 1990; Vol. 184, Chapter 5, pp 51−67.

(4) Bayer, E. A.; Ben-Hur. H.; Wilchek, M. In *Methods in Enzymology;* Wilchek, M., Bayer, E. A., Eds.; Academic Press: San Diego, 1990; Vol. 184, Chapter 8, pp 80−87.

(5) Cuatrecasas, P.; Anfinsen, A. *Annu. Rev. Biochem.* **1971**, *40*, 259.

(6) Bayer, E. A.; Wilchek, M. *J. Chromatogr.* **1990**, *510*, 3−11.

(7) Henrikson, K. P.; Allen, S. H. G.; Maloy, W. L. *Anal. Biochem.* **1979**, *94*, 366−370.

(8) Jung, L. S.; Nelson, K. E.; Campbell, C. T.; Stayton, P. S.; Yee, S. S.; Perez-Luna, V.; Lopez, G. P. *Sens. Actuators, B* **1999**, *B54*, 137−144.

(9) Jung, L. S.; Nelson, K. E.; Stayton, P. S.; Campbell, C. T. *Langmuir* **2000**, *16*, 9421−9432.

(10) Ding, Z.; Long, C. J.; Hayashi, Y.; Bulmus, E. V.; Hoffman, A. S.; Stayton, P. S. *Bioconjugate Chem.* **1999**, *10*, 395−400.

(11) Olejnik, J.; Sonar, S.; Krzymanska-Olejnik, E.; Rothschild, K. J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7590−7594.

(12) Olejnik, J.; Krzymanska-Olejnik, E.; Rothschild, K. J. *Nucleic Acids Res.* **1996**, *24*, 361−366.

(13) Orr, G. A. *J. Biol. Chem.* **1981**, *256*, 761−766.

(14) Weber, P. C.; Ohlendorf, D. H.; Wendoloski, J. J.; Salemme, F. R. *Science (Washington, D. C.)* **1989**, *243*, 85−88.

(15) Hendrickson, W. A.; Pahler, A.; Smith, J. L.; Satow, Y.; Merritt, E. A.; Phizackerley, R. P. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2190−2194.

10.1021/ac020039h CCC: $22.00
Published on Web 08/16/2002

The nature[14,15] and kinetics[16−18] of biotin binding to streptavidin and avidin are important for affinity separations that use biotin-containing reagents or conjugates. In particular, conjugation to the carboxylic group in the biotin side chain can perturb the interaction with the protein loops, thus lowering the binding constant. On the other hand, a weakened interaction can be useful for increasing the extremely low rate of biotin-containing conjugates release to speed up affinity purification.[6]

Wilbur and co-workers studied dissociation from dissolved streptavidin and avidin of biotinylated derivatives of cyanocobalamin[19] that contained an *N*-methylalanine (sarcosine) linker that was conjugated by an amide bond to the biotin carboxylic group.[20] They found that the presence of the sarcosine linker substantially sped up the displacement rate such that <10% of the biotin-containing derivatives was retained with avidin after a 1-h incubation. An additional benefit of the *N*-methyl amide linkage that makes it suitable for biochemical applications is that it resists the enzyme biotinidase, which otherwise cleaves biotin from peptide conjugates.[20]

We have recently reported on the use of biotin−sarcosine conjugates for the quantitative determination of velocities of several lysosomal enzymes in homogenates from cultured human skin fibroblasts,[21−24] as reviewed.[25] Affinity capture−release was used to purify biotin-containing product conjugates that were detected and quantified by ESI-MS using stable-isotope-labeled internal standards.[21−24,26] This method, which we call ACESIMS, has to accommodate the different chemical nature of the substrate conjugates, e.g., the presence of acid-sensitive glycosidic bonds in some substrates, and lipophilic side chains in others, which require specific protocols for affinity capture−release.

The next step in transferring the ACESIMS technology from the research chemical laboratory to clinical analytical practice requires protocol standardization and automation of as many analytical steps as practical. Here we report an automated procedure for affinity capture−release that allows kinetic measurements of dissociation rates of model biotin-containing conjugates (denoted as BPN and BSN, Figure 1) which are tagged with a *p*-nitrophenylthioalkyl chromophore for UV/visible monitoring and contain poly(ethylene glycol)diamine linkers for efficient ionization and detection by ESI-MS. BSN contains a sarcosine linker as in the enzyme substrates described above to mimic their affinity−elution characteristics. An automated ACESIMS procedure is also reported that achieves fast, repetitive, and quantitative determination of β-galactosidase activity in homogenates from cultured human skin fibroblasts.

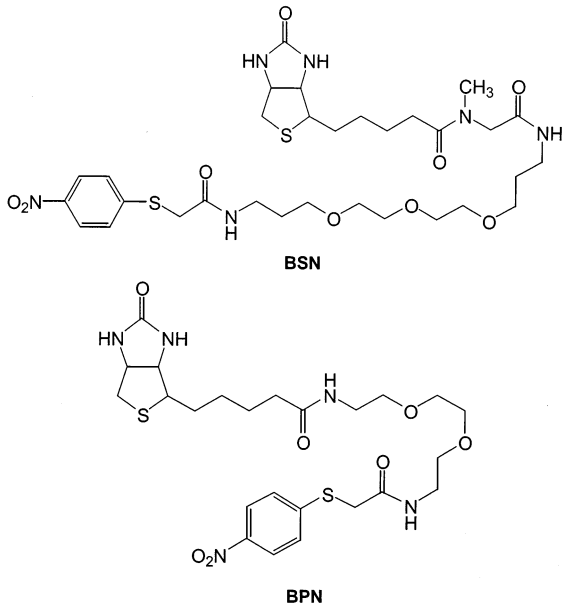

Figure 1. Chemical structures of BSN and BPN.

EXPERIMENTAL SECTION

Materials. 1-Biotinyl-10-(4′-nitrophenylthiomethylcarbonyl)-1,10-diaza-4,7-dioxadecane (BPN) and 1-(*N*-biotinyl)sarcosinyl-15-(4′-nitrophenylthiomethycarbonyl)-1,15-diaza-5,8,11-trioxapentadecane (BSN) were synthesized by coupling *p*-nitrothiophenol (Aldrich) to the pertinent iodoacetylated biotin derivatives according to the general procedures reported previously.[26] The conjugates were characterized by ^{1}H NMR, ESI-MS, and UV/visible spectroscopy.

BPN: ^{1}H NMR (300 MHz, CD_3CN) δ 8.11 (d, 2H), 7.45 (d, 2H), 4.43 (m, 1H), 4.25 (m, 1H), 3.49 (m, 8H), 3.24 (m, 8H), 3.15 (m, 2H), 2.90 (s, 1H), 2.85 (dd, 1H), 2.77 (dd, 1H), 2.13 (m, 2H), 1.56 (m, 6H); mass spectrum 570.3 $(M + H)^+$; UV/visible(CH_3OH) λ_{max} (ϵ_{max}) 330 nm (6100).

BSN: ^{1}H NMR (300 MHz, CD_3CN) δ 8.13 (d, 2H), 7.47 (d, 2H), 7.19 (br s, 1H), 6.62 (br s, 1H), 4.40 (m, 1H), 4.23 (m, 1H), 3.53 (m, 12H), 3.22 (m, 9H), 3.00 (s, 3H), 2.84 (m, 2H), 2.65 (m, 2H), 2.39 (m, 2H), 1.64 (m, 8H); mass spectrum 713.6 $(M + H)^+$; UV/visible (CH_3OH) λ_{max} (ϵ_{max}) 330 nm (7100).

Anion-exchange resin (AG 1-X4 in acetate form, biotechnology grade) was purchased from Bio-Rad (Richmond, CA). Streptavidin-agarose beads were purchased from Pierce (Rockford, IL) and used as received. The beads were suspended in a buffer in a 1:1 v/v ratio when settled. The bead capacity for specific biotin capture was determined by a UV assay at 330 nm using BPN as follows. A 120-μL aliquot of commercial streptavidin bead suspension was placed in a microfiltration capsule (Micro Bio-Spin, Bio-Rad), and the buffer was removed by centrifugation at 1000 rpm for 5 s. The beads were washed with 200 μL of phosphate-buffered saline (PBS) solution (pH 8.9), and the buffer was removed by centrifugation. A 200-μL aliquot of 70 mM BPN in PBS was applied to the beads and incubated at room temperature for 10 min. The supernatant was collected by centrifugation at 1000 rpm for 5 s, the beads were washed twice with 200 μL of PBS, and the filtrates

(16) Green, N., M. *Biochem. J.* **1963**, *89*, 585.
(17) Green N. M. *Biochem. J.* **1966**, *101*, 774.
(18) Piran, U.; Riordan, W. J. *J. Immunol. Methods* **1990**, *133*, 141−143.
(19) Wilbur, D. S.; Chyan, M.-K.; Pathare, P. M.; Hamlin, D. K.; Frownfelter, M. B.; Kegley, B. B. *Bioconjugate Chem.* **2000**, *11*, 569−583.
(20) Wilbur, D. S.; Hamlin, D. K.; Pathare, P. M.; Weerawarna, S. A. *Bioconjugate Chem.* **1997**, *8*, 572−584.
(21) Gerber, S. A.; Scott, C. R.; Tureček, F.; Gelb, M. H. *J. Am. Chem. Soc.* **1999**, *121*, 1102−1104.
(22) Scott, C. R.; Gerber, S. A.; Tureček, F.; Gelb, M. H. *Am. J. Hum. Genet.* **1999**, *65*, 2434 (Suppl. S).
(23) Gerber, S. A.; Scott, C. R.; Tureček, F.; Gelb, M. H. *Anal. Chem.* **2001**, *73*, 1651−1657.
(24) Zhou, X.-F.; Tureček, F.; Scott, C. R.; Gelb, M. H. *Clin. Chem.* **2001**, *45*, 874−881.
(25) Tureček, F. *J. Mass Spectrom.* **2002**, *37*, 1−17.
(26) Gerber, S. A.; Tureček, F.; Gelb, M. H. *Bioconjugate Chem.* **2001**, *12*, 603−615.

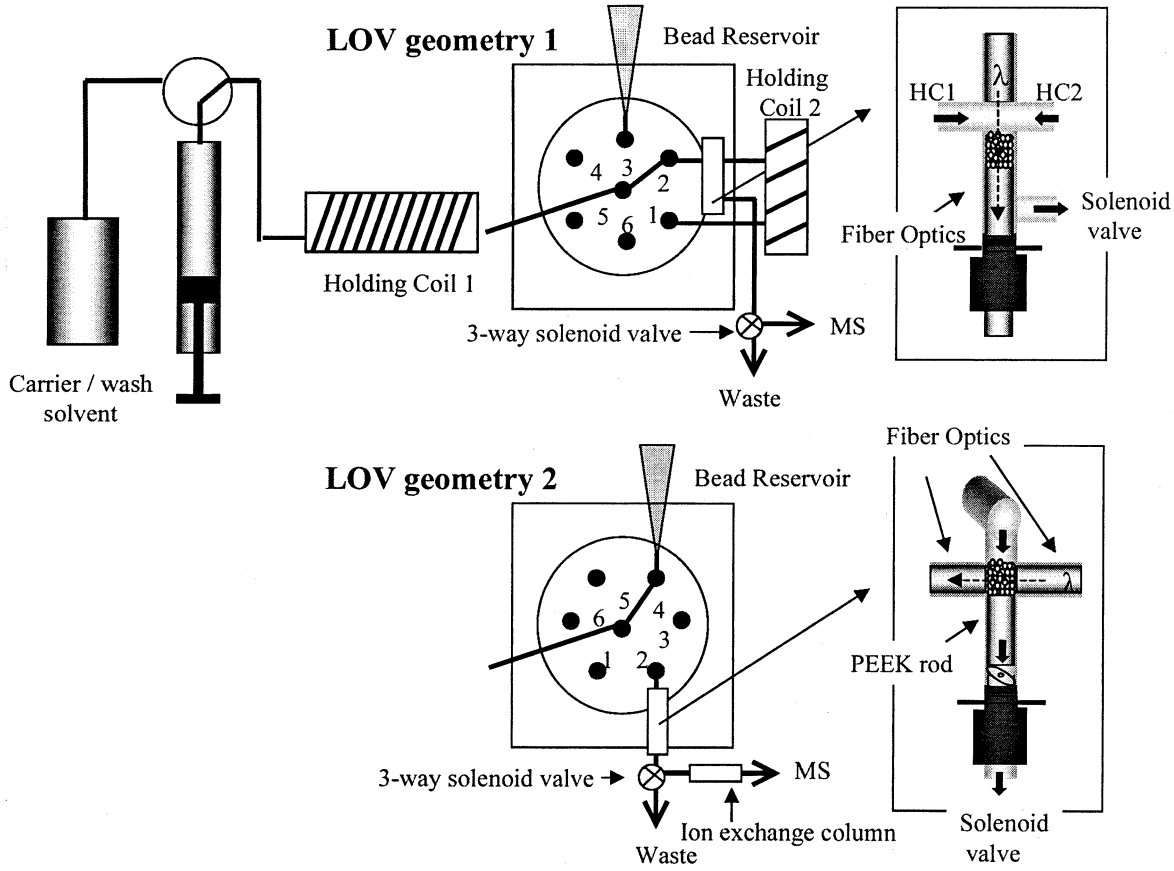

Figure 2. Drawing of the lab-on-valve apparatus with geometry 1 and geometry 2 configurations of the bead-holding cell. LOV geometry 1: port 1, inlet to holding coil 2; port 2, inlet to bead-holding chamber; port 3, bead reservoir; port 4, eluent port (free biotin in methanol/water); port 5, sample; port 6, bead waste. LOV geometry 2: port 1, bead waste; port 2, bead-holding chamber; port 3, not used; port 4, bead reservoir; port 5, eluent port (biotin in methanol/water); 6, sample. HC1 and HC2 designate solvents lines connected to holding coils 1 and 2, respectively. Rotation of the six-port valve connects the central port to any one of the six surrounding ports. The solid arrows in the flow channels and the dotted arrows indicate solvent flow and the direction of UV light, respectively.

were combined. The filtrate volume was adjusted to 1 mL, and its absorbance was measured at 330 nm. The BPN retention on beads was calculated by comparing the filtrate absorbance with that of a control that underwent the same procedure but without beads. The mean bead capacity was calculated from two measurements as 57 pmol/μL of bead buffer suspension or 114 pmol/μL of settled beads.

Methods. The FIAlab-3000 lab-on-valve system was purchased from FIAlab Instruments Inc. (Bellevue, WA) and was controlled by the FIAlab 5.0 software. The UV detection system used a model UV-2 deuterium lamp (Analytical Instrument Systems, Inc., Flemington, FL), an SD 2000 fiber-optic spectrometer (Ocean Optics, Inc., Dunedin, FL), and two 600-mm-diameter ($^1/_{16}$-in. or 1.587-mm diameter including cladding) optical fibers (Ocean Optics). The three-way isolation solenoid valve was purchased from NResearch Inc. (West Caldwell, NJ). All connecting $^1/_{16}$-in.-o.d. tubing was 0.02- or 0.03-in.-i.d. FEP Teflon (Upchurch Scientific, Oak Harbor, WA). Electrospray mass spectra were recorded on a Sciex API III instrument (Sciex, Foster City, CA), using the Tune 2.5 and MacSpec 3.3 data acquisition systems. UV spectra were also taken on a Hewlett-Packard 8452A diode array spectrophotometer.

LOV-ESI-MS Apparatus. The automated instrument for capture, purification, release, and analysis of biotin-containing conjugates was developed from the lab-on-valve (LOV) apparatus designed by Ruzicka for UV/visible spectrophotometry.[27] The modified instrument (Figure 2) consists of a reversible-flow syringe pump, a holding coil, a rotatable six-port valve with a fiber-optics light source and a detector, a bead-holding compartment, a three-way solenoid valve, an ion-exchange microcolumn, and a transfer line to ESI-MS (Figure 2). Sample and bead aspiration, delivery, and disposal are achieved by combinations of reversible-flow pump motion and switching the six-position valve. The ports on the rotatable valve are assigned as shown in Figure 2.

Two LOV geometries were used as shown in Figure 2: both geometries 1 and 2 for kinetic measurements and geometry 2 only for β-galactosidase enzyme activity assays. In geometry 1, the solution holding coil 2 was introduced and mounted adjacent to the bead-holding chamber so that the eluent (free biotin solution) travels a short distance (~2 mm) to reach the beads. In this way, the eluent is not dispersed by laminar flow, and the concentration of the free biotin solution stays uniform during the kinetic measurements. In geometry 1, the six-position valve is mounted such that the optical fiber lines are vertical. The bottom (detector) end of the optical fiber is 4 mm from the end of the upper fiber. This creates a cavity large enough to hold 6 μL of bead suspension in the 1.613-mm-diameter channel. The lower 1.587-mm-o.d. optical fiber functions as a filter plug that holds the beads in the cavity

(27) Růžička, J. *Analyst* **2000**, *125*, 1053−1060.

while allowing the solvent to flow through the bead bed and the coaxial space between the fiber and the channel walls.

In geometry 2, there is no extra holding coil and the eluent is directly pulled from the reservoir. In this geometry, one observes more gradual onset of the eluent zone when applied to the beads. However, in geometry 2, the purification steps are simplified and the analysis time is shortened, which is important for more rapid, repetitive β-galactosidase assay measurements, as described below. In geometry 2, the LOV is mounted such that the optical fibers are horizontal. The beads are retained in the space between the ends of the optical fibers and by a short piece of a $^1/_{16}$-in.-o.d. PEEK rod that is loosely inserted into the vertical channel leading to the three-way isolation valve. The PEEK rod serves as a filtration plug that prevents the passage of beads but allows solution to flow through the bead bed.

The general operation sequence of the LOV consists of several computer-controlled liquid and bead injections. Step 1: The syringe pump aspirates a 1-mL volume of the wash solvent (50/50 methanol/water) and uses 400−500 μL to flush the bead-holding chamber twice in the LOV. The remaining liquid volume is kept in holding coil 1 and serves as a liquid piston for further bead and sample aspiration and delivery. For geometry 1, 50 μL of eluent (free biotin solution) is sent to holding coil 2 and is stored there until elution (step 5, see below). Step 2: A small volume of the streptavidin-agarose bead suspension (typically 6−10 μL) is aspirated from the bead reservoir and delivered to the bead-holding compartment (Figure 2). Step 3: A 100−200-μL volume of sample in 50/50 methanol/water is aspirated from the sample reservoir through one of the available ports on the LOV into holding coil 1. The syringe pump is then reversed to perfuse the sample through the bead compartment. Note that the dead volume in the bead compartment is <5 μL, as estimated from close packing of spherical beads. Thus, a 100−200-μL volume of sample solution achieves 20−40-fold perfusion of the bead compartment volume, which results in very efficient affinity capture, as discussed below. Sample solution exits the bead compartment and is routed through the three-way solenoid valve to waste. Step 4: The beads with captured biotin-containing conjugates are washed with ~200 μL of wash solution to remove nonspecifically bound analytes, and the wash solution is also directed through the three-way solenoid valve to waste. These affinity cleanup steps can be conveniently monitored by absorbance measurements of beads with the fiber-optic system. Step 5: Eluent (a solution of free biotin or a biotin derivative, if needed)[24] is aspirated by the syringe pump through one of the remaining LOV ports and perfused through the beads to release the captured biotin-containing conjugates. The sample depletion is simultaneously monitored with UV/visible spectrometry. Note that, for geometry 1, the eluent is delivered through holding coil 2. The solution with the released conjugates is delivered through the three-way solenoid valve to an on-line ion-exchange microcolumn that retains free biotin and further on to the ESI-MS for analysis. Step 6: The bead-holding compartment is filled with wash solvent and the flow is reversed, such that the beads are lifted out of the bead compartment, and the flow is reversed again to force the beads through the waste port (i.e., port 6, Figure 2, top). The system is now ready for the next cycle starting with the washing step (step 1). Note that the sequence, timing, and volumes used in each full cycle are entered as parameters in the data system and executed automatically by the FIAlab software. A complete sequence of pump and valve operations is given in Table S1 (Supporting Information).

RESULTS AND DISCUSSION

The LOV apparatus with geometry 1 and geometry 2 configurations was used for measurements of affinity capture, release kinetics, and enzyme assay. These different applications are discussed separately.

Capture and Elution Efficiency Measurements. The efficiency of capturing and releasing biotin-containing conjugates on streptavidin-agarose beads was measured for BSN at several BSN−streptavidin molar ratios using the LOV apparatus in the geometry 2 configuration. In each measurement, a 10 μM solution of BSN in 50/50 (v/v) methanol/water was perfused at a 1 μL/s flow rate through the beads, such that the injected volumes were 25, 50, 100, and 150 μL. This corresponds to delivering 250, 500, 1000, and 1500 pmol of BSN, respectively, to be captured by 670 pmol (the capturing capacity) of immobilized streptavidin. Hence, the protein was exposed to substoichiometric, approximately stoichiometric, and excessive amounts of BSN. The flow-through from the beads was collected, and the beads were washed with 50/50 methanol/water to remove nonspecifically bound conjugates. The flow-through and the wash solution were combined and quantified for BSN by ESI-MS using BPN as an internal standard. The relative ESI-MS response to BSN and BPN was obtained from a calibration curve measured for standard mixtures of BSN and BPN in 1.0, 0.5, 0.25, and 0.1 mol ratios. A linear response was obtained with a correlation coefficient of $r^2 = 0.9998$. The measurements showed that, for solutions with substoichiometric amounts of BSN, 78% was affinity captured on the beads on average. With stoichiometric and excess BSN, the capture efficiency was 90−100% of bead capacity. The small difference in the capture efficiency may be due to mass-transfer kinetics as the beads were exposed to the BSN solution for longer times at higher molar loadings.

ESI-MS was also used to measure the elution efficiency using 400 μL of a 45 μM solution of free biotin in 50/50 methanol/water corresponding to a total of 18 nmol of biotin. Under these conditions of excess biotin over streptavidin, the elution of BSN was nearly complete (>95%). In summary, the capture−release of BSN shows a very good overall efficiency, which is important for kinetic measurements and sensitive enzyme assays, as described later.

Elution Kinetics of BSN and BPN. Elution of BSN and BPN with a 60 μM solution of free biotin was readily monitored by measuring the decrease of analyte absorbance at 330 nm on the beads with the fiber optics, in combination with ESI-MS detection and quantification of the analyte eluted in solution. The elution curves showed very different slopes that indicated much faster elution of BSN due to the presence of the sarcosine linker (Figure S1, Supporting Information).[19] Figure 3 shows the elution curves of BSN at different concentrations of free biotin in 50/50 methanol/water. In the absence of biotin, BSN is eluted only very slowly from the beads. In contrast, at biotin concentrations of ≥30 μM, elution approaches 100% within 100 s.

The elution curves obtained concurrently by ESI-MS show qualitatively similar characteristics (Figure S2, Supporting Infor-

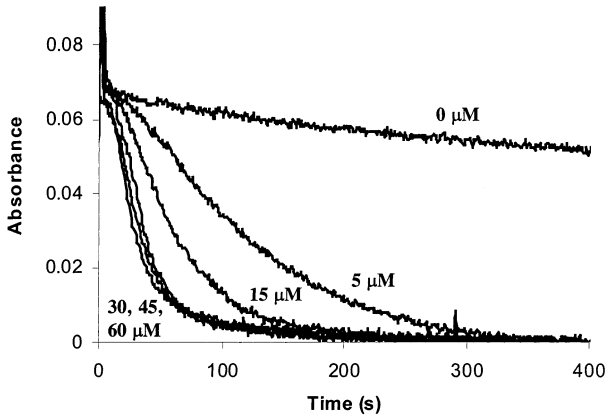

Figure 3. Elution curves of BSN measured by UV/visible spectrometry at the indicated concentrations of free biotin in (50/50) methanol/water.

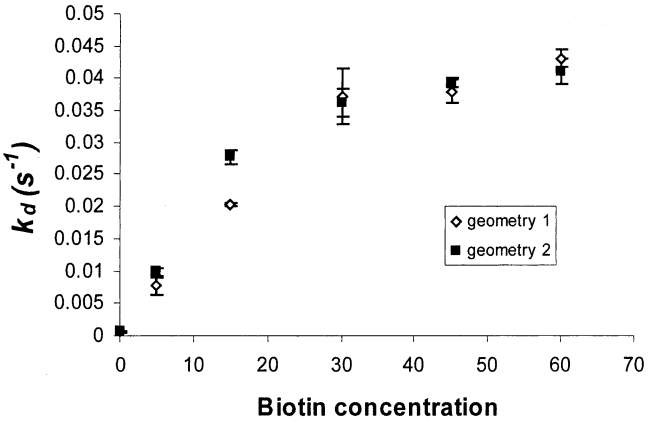

Figure 4. Apparent dissociation rate constants (k_d) for BSN as a function of biotin concentration.

mation). At biotin concentrations of ≥30 μM, the ESI-MS elution profiles remain virtually constant and indicate fast BSN elution. However, the ESI-MS elution curves show tails up to ~200 s of elution time due to zone broadening by laminar flow from the bead space to the ESI interface. Note that the absorbance measurements (Figure 3) monitor the decrease of the molar amount of streptavidin-bound BSN because the bead volume remains constant during elution. In contrast, ESI-MS monitors the instantaneous concentration of BSN flowing into the ESI interface. Hence, integration of the ESI-MS elution profiles provides curves, which are convolutions of the UV/visible elution curves and the zone-broadening function.

The BSN-streptavidin elution kinetics was modeled as a first-order dissociation. This is based on the assumption that reassociation of BSN with streptavidin is negligible because the released BSN is removed by solvent flow and the vacated binding sites on streptavidin are occupied by free biotin. The dissociation rate is expressed by eq 1, where R_t is the response (absorbance in UV/visible measurements), k_d is the apparent dissociation rate constant, and t is time.

$$dR_t/dt = -k_d R_t \quad (1)$$

Integration of eq 1 gives eq 2, where R_{t_1} and R_{t_n} are the responses measured at times t_1 and t_n, respectively, which allows evaluation of k_d by linear regression of $\ln(R_{t_1}/R_{t_n})$ versus time.

$$\ln(R_{t_{1n}}/R_{t_n}) = k_d(t_n - t_1) \quad (2)$$

Elution curves consisting of several hundred data points were measured for biotin concentrations of 5, 15, 30, 45, and 60 μM, and the measurements were repeated three times for each concentration. Values of k_d, for BSN at several biotin concentrations are summarized in Figure 4. The data show a very slow apparent dissociation rate constant ($k_d < 10^{-4}$ s^{-1}) in the absence of biotin and increasing k_d that approaches a limit value of 0.04 s^{-1} at ≥30 μM biotin concentrations. The saturation effect on the dissociation kinetics shown in Figure 4 is probably due to biotin binding. At high biotin concentrations, the BSN released from the beads is replaced rapidly by free biotin, so that the overall process follows zero-order kinetics with respect to biotin and first-order kinetics with respect to the bound BSN (eq 1). At low biotin concentrations, BSN recapture competes with biotin binding and the overall kinetics is approximately first order with respect to free biotin, as shown by the initial portion of the curve in Figure 4. For practical purposes, the kinetic measurements provide an estimate of the lowest biotin concentration that achieves the limit dissociation rate.

The dissociation rate constant for BPN is notably slower than the limiting k_d for BSN. At 60 μM biotin concentration, the k_d for BPN was measured at $(1.7 \pm 0.3) \times 10^{-3}$ s, which is over 20 times lower than the value for BSN. This indicates that biotinylated conjugates incorporating the sarcosine linker are to be preferred if fast release from streptavidin beads is required. Interestingly, the k_d value for BPN measured here was substantially greater than the rate constants measured previously for free biotin[18] and its conjugates.[8,9] One reason for this difference may be due to the use of 50/50 methanol/water, as opposed to phosphate-buffered saline (PBS) used previously. To test this hypothesis, we re-measured BPN elution with 90 μM biotin in 50/50 methanol/water and PBS with UV/visible monitoring (Figure S3, Supporting Information). This showed that BPN elution was 3 times faster in methanol/water, confirming that solvent composition affects the dissociation kinetics.

***β*-Galatosidase Assay in Cell Homogenates.** One of the goals of the present study was to develop a practical automated procedure for clinical measurements of enzyme velocities in complex biological mixtures such as cultured cell homogenates. This is demonstrated by an assay of lysosomal β-galactosidase in human skin fibroblasts. β-Galactosidase is an enzyme that cleaves the terminal galactose from the complex sphingolipid GM_1 ganglioside. When the enzyme is defective because of an inborn error, the lack of activity results in accumulation of unprocessed GM_1 ganglioside in the lysosome and causes the rare autosomal recessive disorder GM_1 gangliosidosis in affected children.[28] β-Galactosidase is assayed by a synthetic biotin-containing conjugate described previously.[21] Action of the enzyme causes cleavage of galactose and results in a change of mass by 162 Da,

(28) Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Eds. *The Metabolic and Molecular Bases of Inherited Disease*, 8th ed.; McGraw-Hill: New York, 2001.

which is readily detected by ESI-MS. Quantification is achieved by measuring the response of the product conjugate at m/z 681 relative to that of an isotope-labeled internal standard at m/z 689. The internal standard is chemically identical to the enzymatic product but is distinguished by mass due to the presence of eight deuterium atoms in the linker.[21] The chemical similarity of the product conjugate and internal standard ensures their identical behavior in binding to and release from streptavidin, loss of material due to nonspecific binding to various surfaces in the reaction tube and LOV apparatus, and ionization and detection efficiencies in ESI-MS.

The LOV-ESI-MS apparatus for β-galactosidase assay was used in the geometry 2 configuration, which was slightly modified by removing the optical fibers and capping the corresponding ports. The general enzyme assay procedure is described elsewhere[21,23] and was followed here with a few changes. The operation sequence included aspiration of streptavidin-agarose beads (10 μL) through port 4 and injection into the bead-holding chamber. Next, the cell supernatant containing the unreacted biotin-containing substrate conjugate, the product, and the internal standard was aspirated from port 6 and injected at 10 μL/s into the bead chamber for affinity capture. The beads were washed with (50/50) methanol/water at 25 μL/s to remove nonspecifically bound cell components, and the wash solution was discarded to waste. Release from beads was accomplished with 100 μL of 45 μM biotin solution containing 0.03% acetic acid to facilitate protonation of conjugates in ESI. The released biotin-containing conjugates were passed through the on-line ion-exchange column and injected into the ESI interface at a flow rate of 1 μL/s. Mass spectra were monitored in the regions of m/z 675−697 for the product conjugate and internal standard and of m/z 840−848 for the substrate conjugate. The resulting ESI-MS spectra are shown in Figure 5. The top spectrum shows a blank assay that contained the substrate conjugate and internal standard but lacked cell lysate. Product formation by nonenzymatic processes, e.g., nonspecific hydrolysis, ion dissociation in the interface, etc., is negligible. The bottom spectrum shows an assay using a lysate from cells from a healthy individual. Formation of the product is clearly indicated by the $(M + H)^+$ peak at m/z 681. The [product]/[internal standard] ratios from repetitive measurements were reproducible within ±3% (one standard deviation) for four repetitive measurements of lysate aliquots. The calculated β-galactosidase velocities are summarized in Table 1 for seven different enzyme assay batches that were carried out with cultured skin fibroblasts from the healthy donor. For each batch, the sample was injected four times into the LOV apparatus. The intrabatch reproducibility (error in the range of 0.5−1.7) was better than the error between the batches (3.8). The latter reflects errors in aliquot volume measurements that were done by manual pipeting. The full analytical cycle including bead removal and washing takes 4.5 min. By comparison, the same operation takes 1−6 h (for elution alone) plus pipeting time when done manually in a batch mode by a skilled operator.[29]

The robustness of the LOV apparatus for the β-galactosidase assay was tested by running 60 automatic repetitive injections in which each analytical cycle was performed within 4.5 min. On the basis of the flawless operation in 60 injections, we expect that it should be possible to operate the LOV-ESI-MS apparatus using an autosampler in an unattended automatic run. Efforts to this end are in progress in this laboratory.

(29) Gerber, S. A. Ph.D. Thesis, University of Washington, 2001.

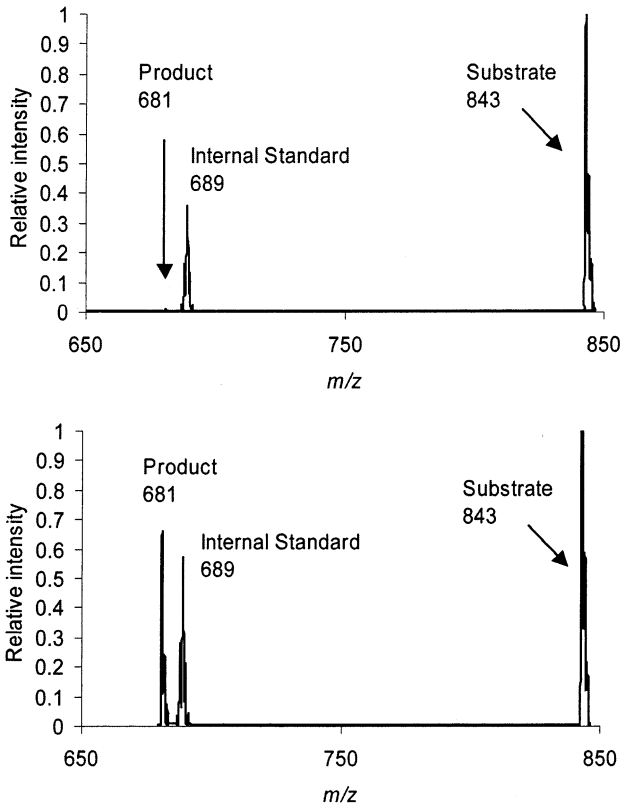

Figure 5. Electrospray ionization mass spectra of affinity-purified eluates from (top) blank and (bottom) assay of β-galactosidase in cultured human skin fibroblasts.

Table 1. β-Galactosidase Activity Measurements in Cultured Human Skin Fibroblasts

	sample[a]								
	1	2	3	4	5	6	7	mean	blank[b]
enzyme velocity[c]	49.2	48.1	45.5	46.4	42.8	46.1	44.4	46.1	0.2
sample standard deviation[d]	1.7	1.3	1.3	1.0	1.2	1.1	0.5	3.8[e]	0.05

[a] Different enzyme assay batches using cells from the same healthy donor. [b] Nonenzymatic product formation in the absence of cells. [c] In units of [nmol of product] h^{-1} [mg of protein]$^{-1}$. [d] From four repetitive measurements of multiple injections of the same sample. [e] Standard deviation of the mean from seven incubation batches.

CONCLUSIONS

The combination of affinity capture−release electrospray ionization mass spectrometry with automated sample handling by a lab-on-valve apparatus equipped with UV/visible detection provides in situ monitoring of the kinetics of interaction between biotin-containing conjugates and immobilized streptavidin. The power of this approach is demonstrated by detailed kinetic analysis of dissociation of chromophore-tagged biotin conjugates that revealed the effects of solvent and biotin concentration. In addition, the apparatus was shown to perform well in analysis of enzyme velocities in cultured human skin fibroblasts using on-line coupling

to an electrospray ionization mass spectrometer. The short time of analysis (4.5 min) and high repeatability (60 samples) are very promising for transferring LOV-ESI-MS to the clinical analytical laboratory.

ACKNOWLEDGMENT

This project was supported by grants from the National Institutes of Health (GM 60184 to M.H.G., C.R.S., and F.T., and GM 45260 to J.R. and L.S.). Funding for the Bruker Esquire instrument was provided jointly by the National Science Foundation CRIF Program (CHE-9807748) and University of Washington. Thanks are due to Dr. Scott Gerber for assays, Dr. Martin Sadílek for assisting with mass spectrometric measurements, and Dr. Rhona Jack and Ms. Nancy McDowell for preparation of cell cultures.

SUPPORTING INFORMATION AVAILABLE

Figures of elution curves (Figures S1−S3) and Table S1 with a complete list of syringe and valve switching operations. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review January 22, 2002. Accepted June 7, 2002.

AC020039H