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sensitive, accurate and reproducible. With complete DNA sequences becoming available for various organisms, including those from the human genome project, increasing emphasis is being placed on determining the function of unknown genes. A fundamental prerequisite in assigning function is to establish whether a protein is secreted or remains inside the cell. This can now be readily accomplished by replacing gelatinase A in the GFR construct described above with the unknown gene to be characterized. One qualifying comment should be made. Secretory RNase H activity will also be observed in the case of plasma membrane proteins in which the C terminus is extracellular.

REFERENCES

1. Ailenberg, M. and M. Silverman. 1996. Cellular activation of mesangial gelatinase A by cytochalasin D is accompanied by enhanced expression of both gelatinase A and its membrane-associated gelatinase A activator (MT-MMP). *Biochem. J.* 313:879-884.
2. Ailenberg, M. and M. Silverman. 1996. Description of a one step staggered reannealing method for directional cloning of PCR-generated DNA using sticky-end ligation without employing restriction enzymes. *Biochem. Mol. Biol. Int.* 39:771-779.
3. Ailenberg, M. and M. Silverman. 1997. Site-directed mutagenesis using a PCR-based staggered re-annealing method without restriction enzymes. *BioTechniques* 22:624-630.
4. Ailenberg, M., T. Weinstein, I. Li and M. Silverman. 1994. Activation of procollagenase IV by cytochalasin D and concanavalin A in cultured rat mesangial cells: linkage to cytoskeletal reorganization. *J. Am. Soc. Nephrol.* 4:1760-1770.
5. Alam, J. and J.L. Cook. 1990. Reporter genes: application to the study of mammalian gene transcription. *Anal. Biochem.* 188:245-254.
6. Denault, J.-B. and R. Leduc. 1996. Furin/PACE/SPC1: a convertase involved in exocytic and endocytic processing of precursor proteins. *FEBS Lett.* 379:113-116.
7. Groskreutz, D., M.X. Sliwkowski and C.M. Gorman. 1994. Genetically engineered proinsulin constitutively processed and secreted as mature, active insulin. *J. Biol. Chem.* 269:6241-6245.
8. Liu, Y.-C., M. Kawagishi, T. Mikayama, Y. Inagaki, T. Takeuchi and H. Ohashi. 1993. Processing of a fusion protein by endopeptidase in COS-1 cells for secretion of mature peptide by using a chimeric expression vector. *Proc. Natl. Acad. Sci. USA* 90:8957-8961.
9. Lu, Z., E.A. DiBlasio-Smith, K.L. Grant, N.W. Warne, E.R. La Valle, L.A. Collins-Racie, M.T. Follettie, M.J. Willington and J.M. McCoy. 1996. Histidine patch thioredoxins. Mutant forms of thioredoxin with metal chelating affinity that provide for convenient purification of thioredoxin fusion proteins. *J. Biol. Chem.* 271:5059-5065.
10. Pei, D. and S.J. Weiss. 1995. Furin-dependent intracellular activation of the human stromelysin-3 zymogen. *Science* 375:244-247.
11. Steiner, D.F., S.P. Smekens, S. Ohagi and S.J. Chan. 1992. The new enzymology of precursor processing endoproteases. *J. Biol. Chem.* 267:23435-23438.
12. Watanabe, T., K. Murakami and K. Nakayama. 1993. Positional and additive effects of basic amino acids on processing of precursor proteins within the constitutive secretory pathway. *FEBS Lett.* 320:215-218.

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Menachem Ailenberg and Mel Silverman

*University of Toronto
Toronto, ON, Canada*

On-Line Microdialysis for Mass Spectrometry

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New ionization techniques such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) allowing the examination of large biomolecules by mass spectrometry have catapulted the method to the center of microbiological and biochemical research. However, common contaminants in biological samples (polyethylene glycol [PEG], buffer salts, dioctyl phthalate etc.) present background that often results in a partial or

complete loss of signal from the molecule of interest. Several off-line dialysis devices are available, providing dialysis or ultrafiltration of microliter samples prior to mass spectrometric analysis. These include dialysis cassettes (Pierce Chemical, Rockford, IL, USA), floating membranes (Millipore, Bedford, MA, USA) and centrifugal filters (Orbital Biosciences, Topsfield, MA and Chemicon International, Temecula, CA, USA). However, they do have some disadvantages with regard to mass spectrometry in terms of dialysis rate and recovery, and most are not easily reusable. Direct, on-line dialysis overcomes these problems and is still desirable for many applications. Flow microdialysis devices have been available for several years for sampling low-molecular-weight compounds from living systems (Harvard Apparatus, Holliston, MA, USA). Wu et al. (4) have applied the reverse of this principle to on-line desalting of proteins for ESI mass spectrometry. Their idea is based on the use of only one of the 176 fibers of the Fleaker® Hollow Fiber Concentration System (Spectrum Medical Industries, Laguna Hills, CA, USA). These fibers consist of regenerated cellulose and have a molecular weight cut-off of 13 000 Da. The fiber is connected on both ends to fused silica tubing (100-µm inner diameter [id]/186-µm outside diameter [od]) and placed inside of a flexible Teflon® tube with Swagelok™ T Connectors (Swagelok, Solon, OH, USA) at both ends, arranged to allow buffer to flow on the outside of the fiber. The same laboratory also described a similar microdialysis junction for capillary electrophoresis mass spectrometry (2).

Impressed by this work, we constructed the device described in Reference 4 for use in conjunction with a Model TSQ 700 Triple Quadrupole Mass Spectrometer (Finnigan MAT, San Jose, CA, USA), and we can confirm its great effectiveness in dialyzing proteins on-line. However, we found that construction of the device, although straightforward, is somewhat difficult in that rotation of the epoxy-glued joints occurs as the compression nuts on the T connectors are tightened. As a result, the fragile connections tend to break or cut through the cellulose. In

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this paper, we suggest an alternate construction of the more compact and rugged design shown in Figure 1.

To prepare it for use, its two halves are separated as shown, the epoxy-glued membrane with its fused silica capillary joints inserted in the longitudinal groove, the gasket aligned and the halves reassembled. Special care must be taken during its first assembly not to crush the cellulose fiber between the blocks, but once it is safely mounted inside, it is very stable. The dialysis solvent runs in counterflow by gravity alone; there is no need for a more controlled flow, and one pump is saved. After assembly, the integrity of the sample line should be examined using a dye whose path inside the fiber can be followed. The translucent Plexiglas® facilitates this procedure. The outlet capillary of the device is connected with a short piece of Teflon tubing directly to the inlet capillary of the mass spectrometer source so that all low-molecular-weight impurities are removed prior to entering the mass spectrometer. The device should be stored in the refrigerator if not used for extended periods of time to prevent bacterial growth. Lamoree et al. (1) have recently described a similar system with a much shorter dialysis region (3 mm) for use with capillary electrophoresis mass spectrometry.

For protein studies, we often dialyze against 5% acetic acid (Mallinckrodt Baker, Paris, KY, USA), 95% 1/1 (vol/vol) methanol (Mallinckrodt Baker)/HPLC-grade water (Fisher Scientific, Pittsburgh, PA, USA) because this is a convenient solvent for ESI mass spectrometry. Typical flow rates are 3–5 $\mu\text{L}/\text{min}$. Figure 2 shows an example of the effectiveness of the system. The sample was a routine molecular weight determination for quality control of the recombinant protein ^{13}C , ^{15}N -labeled vnd/NK-2 mutant homeodomain (3). However, we were not aware of the fact that the sample contained a large amount of PEG as indicated in Figure 2 (upper spectrum) by the multitude of peaks separated by 44 atomic mass units ($\text{CH}_2\text{CH}_2\text{O}$). Redirection of the sample flow through the dialysis device led to the lower spectrum, which now clearly shows the protein charge envelope along with some residual PEG,

whose actual amount can be controlled further by optimizing the dialysis parameters. It is of interest that the vnd/NK-2 mutant protein, having a molecular weight of 10 215 Da, could be determined even though the molecular weight cut-off of the fiber is 13 000 Da. On further examination, using wild-type NK-2 protein (molecular weight 9695 Da), we found that we had in fact lost about half of the protein (protein concentrations were determined by the bicinchoninic acid assay method [Pierce Chemical]).

The system can also be used to concentrate the protein inside the dialysis fiber. This may be of value in cases in which the amount of protein available is very low or too dilute. As described by Wu et al. (4), a low sample flow (ca. 1 $\mu\text{L}/\text{min}$) actually keeps the protein from exiting even after long times. This was attributed to the loss of elution solvent through the membrane into the dialysis solvent. However, we have found that adsorption of the protein to the walls of the fiber is also a factor. By using ^{14}C -

labeled bovine serum albumin (Sigma Chemical, St. Louis, MO, USA) and counting the solution before and after passage through the dialysis fiber, we found the amount of adhering protein to be 10 fmol/cm of length. As a lower limit of the amount of protein necessary, we determined that we are able to dialyze and measure 10 ng of apomyoglobin (Sigma Chemical) loaded onto the fiber in 10 μL solution.

Many samples brought to us from biochemical and microbiological laboratories are proteins whose concentration and composition are often not well known. Taking advantage of the above features of the device, we typically examine their response by first eluting a very small portion at a low sample flow rate (1–2 $\mu\text{L}/\text{min}$), monitoring the output with the spectrometer. If no signal or only a weak signal is observed, the syringe is refilled with dialysis buffer and the flow rate sharply raised to 3–5 $\mu\text{L}/\text{min}$, causing the trapped protein to elute in a low volume, maximizing the spectrometer signal.

Figure 1. Schematic of microdialysis device. It consists of two Plexiglas blocks of equal size (11: lower part, length 15 cm, width 4 cm, height 1.5 cm). Not shown in the drawing are ten 3-cm-long 1/8-in screws (5 on each side, 1 cm from the groove and distributed over the whole length), which hold the parts firmly together. Both blocks have half of a channel along their midline corresponding to a total id of 4 mm when assembled. One centimeter from either end, the channel narrows to 1/16 in to form a seal with 1/16-in Teflon tubing. The top block contains two holes (1.5 cm from either end) threaded for finger-tight fittings (Upchurch Scientific, Oak Harbor, WA, USA), which extend down to the channel and provide a conduit for the dialysis solvent. The Teflon tubing used for the solvent line is 1/16-in od and only slightly less id (0.038 in) to maximize the solvent throughput. The assembly of the dialysis line follows the instructions in Reference 4, in which fused silica capillary 4 is slid approximately 1 cm into hollow fiber 5, for the epoxy glue junction 6. Teflon tubing 3 (0.007-in id, 1/16-in od) is used for mounting into the ends of the channel and sealing.

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Figure 2. ESI mass spectrum of vnd/NK2 recombinant protein (relative intensity vs. m/z). Top: PEG-containing sample. Bottom: after dialysis against 5% acetic acid, 50/50 (vol/vol) methanol/water. The small peaks on the high mass sides of the main charge states are due to traces of phosphate. The ion at m/z 625 is a triple-charged peptide impurity of 1872 Da.

One disadvantage of this system is that cellulose fibers are not available at the lower-molecular-weight cut-offs required for peptides or smaller proteins. One alternative may be the microdialysis probes for *in vivo* studies (Harvard Apparatus), which offer a molecular weight cut-off of 6000 Da, but they are intended for one-time use and are not as robust. The dialysis tubing used by Lamoree et al. (1) in conjunction with their design for isoelectric focusing was constructed from an artificial kidney with a molecular weight cut-off of 5000 Da.

The device described above has been in regular use in our laboratory for

over one year and is the method of choice whenever salt and low-molecular-weight impurities are known or suspected to be present.

REFERENCES

1. Lamoree, M.H., U.R. Tjaden and J. Van der Greef. 1997. Use of microdialysis for the on-line coupling of capillary isoelectric focusing with electrospray mass spectrometry. *J. Chromatogr. A* 777:31-39.
2. Severs, J.C. and R.D. Smith. 1997. Characterization of the microdialysis junction interface for capillary electrophoresis/microelectrospray ionization mass spectrometry. *Anal. Chem.* 69:2154-2158.
3. Weiler, S., J.M. Gruschus, D.H.H. Tsao, L. Yu, L.-H. Wang, M. Nirenberg and J.A. Ferretti. Site directed mutations in the vnd/NK-2 homeodomain: basis of variations in structure and sequence specific DNA binding. *J. Biol Chem.* (In press)
4. Wu, Q., C. Liu and R.D. Smith. 1996. On-line microdialysis desalting for electrospray ionization-mass spectrometry of proteins and peptides. 1996. *Rapid Commun. Mass Spectrom.* 10:835-838.

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**Simone König, Solly Weiler
and Henry M. Fales**
*National Heart, Lung, and
Blood Institute, NIH
Bethesda, MD, USA*