

Research Article

Isolation of Low Abundance Proteins and Cells Using Buoyant Glass Microbubble Chromatography

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Conventional protein affinity chromatography relies on highly porous resins that have large surface areas. These properties are ideal for fast flow separation of proteins from biological samples with maximum yields, but these properties can also lead to increased nonspecific protein binding. In certain applications where the purity of an isolated protein is more important than the yield, using a glass solid phase could be advantageous as glass is nonporous and hydrophilic and has a low surface area and low nonspecific protein binding. As a proof of principle, we used protein A-conjugated hollow glass microbubbles to isolate fluorescently labeled neurofilament heavy chain spiked into serum and compared them to protein A Sepharose and protein A magnetic beads (Dynabeads) using an anti-neurofilament protein antibody. As expected, a greater volume of glass bubbles was required to match the binding capacity of the magnetic beads and Sepharose resins. On the other hand, nonspecific protein binding to glass bubbles was greatly reduced compared to the other resins. Additionally, since the glass bubbles are buoyant and transparent, they are well suited for isolating cells from biological samples and staining them *in situ*.

1. Introduction

Glass is essentially an amorphous 3-dimensional mesh of silica oxides terminating at the surface as silicon hydroxides. Although the surface exposed silica hydroxides readily coordinate with divalent cations such as Ca^{2+} and can promote surface activated plasma coagulation [1], glass is surprisingly resistant to protein adsorption. For example, glass bead chromatography has been used to specifically purify vitronectin, a cell adhesion protein, from serum with a high degree of purity [2]. Vitronectin has an arginine-glycine-aspartic acid (RGD) integrin recognition motif and was initially identified as “serum spreading factor” due to its ability to bind tissue culture plates and mediate cell adhesion and spreading [3]. Additionally, glass beads can be functionalized with a variety of silanes containing amine and thiol functional groups that can be used for biomolecular conjugation [4, 5]. However, development of porous polymer resins with superior flow characteristics and greater surface area have since replaced

glass beads as the solid support of choice for most protein purification.

In this study, we revisit glass chromatography by examining the ability of hollow glass microbubbles to isolate proteins, or cells, from serum and blood. Glass bubbles are inexpensive, lightweight, and strong hollow silica spheres that are commonly used in a variety of industrial applications ranging from paints and sealants to adhesives. Glass bubbles are buoyant and float to the surface of liquids, thus separating readily from the bulk solution. Paradoxically, the worst properties of using glass for protein isolation could be its greatest asset, namely, the very low surface area and lack of porosity.

Serum proteomics is currently striving to characterize protein constituents that could enable the discovery of disease biomarkers. However, this is complicated by the fact that there are numerous plasma proteins, with concentrations spanning at least 9 orders of magnitude, while many proteins of diagnostic value present at low concentrations. To analyze

valuable serum markers, one must account for the approximately 10 major proteins, including serum albumin and immunoglobulins, which account for more than 90% of the total protein content of serum [6, 7]. Strategies to reduce the interference from these abundant proteins include depleting them from the serum sample or enriching the low abundance proteins of interest. Isolation of proteins from this complex mixture is often performed by immunoprecipitation, but conventional resins (i.e., agarose, sepharose, magnetic beads, etc.) often bind proteins nonspecifically and thus require additional purification steps for samples [8].

As a proof of principle for utilizing glass as a solid support for purifying low abundance proteins, we performed immunoaffinity isolation of purified neurofilament heavy chain spiked into low-IgG fetal bovine serum. Neurofilament proteins belong to the intermediate filament family and are major constituents of the neuronal cytoskeleton. They are mostly expressed by large neurons and myelinated axons and play an important role in neuronal structure and intracellular trafficking [9]. The neurofilament scaffolding is mostly composed of a mixture of three neurofilament proteins known as light, medium, and heavy chains that may also include smaller amounts of ancillary proteins (i.e., peripherin, nestin, and vimentin) [10]. Because of their specific localization in the central nervous system (CNS), their presence outside of this compartment may indicate damage and/or disease of the CNS [11]. The presence of neurofilament heavy chain in the cerebrospinal fluid (CSF) is indicative of axonal damage in disorders such as Parkinson's disease, Alzheimer's disease, and HIV dementia [12–15].

Neurofilament heavy chain was fluorescently labeled to follow the purification process. Immunoprecipitation was performed using neurofilament heavy chain antibodies immobilized on protein A-functionalized glass bubbles, protein A-conjugated Sepharose, and protein A-conjugated Dynabeads. Our results show that though glass bubbles have much lower binding capacity than Sepharose and Dynabeads, the glass bubbles had less nonspecific protein binding, as seen by the amount of total protein eluted and by SDS-PAGE protein profile.

In addition to protein purification, glass bubble chromatography was used for cell isolation. Isolation of prostate cancer cells (PC-3) by glass bubbles was shown to be highly specific and allowed for clear fluorescent staining and imaging of cells as the glass bubbles are transparent.

2. Materials and Methods

2.1. Materials. Protein A Sepharose, fluoresceinbiotin, and (3-glycidyloxypropyl)-trimethoxysilane were purchased from Sigma Chemical Company. NeutrAvidin was purchased from Pierce. Polydisperse hollow silica microspheres (5–40 μm diameter) were purchased from 3M. Dynabeads protein A and Ultra Low IgG fetal bovine serum (low IgG-FBS) were purchased from Invitrogen. FluoroLink-Ab Cy5 labelling kit was purchased from GE Healthcare. Silver Stain Kit was purchased from Pierce. Monoclonal antibody against the 200 kD neurofilament heavy chain (no. ab7795)

was purchased from Abcam. Porcine 200 kD neurofilament heavy chain was purchased from Millipore.

2.2. Conjugation of Glass Microbubbles. The glass bubbles were first washed in concentrated sulphuric acid followed by washing with diH_2O . These steps clean the surface of the bubbles and remove both broken and smaller, less buoyant glass bubbles. After drying the glass bubbles, silane and either protein A or Neutravidin were reacted together in methanol for 2–3 hrs at RT. This was followed by washing away unbound reactants and then incubating with a blocking agent.

2.3. Protein Labeling and Immunoprecipitation. Porcine neurofilament heavy chain was labelled with Cy5, and the labeled neurofilament heavy chain was serially diluted into 1 mL of low IgG-FBS starting at 1.24 ng/mL. All protein A resins and glass bubble slurries were incubated with 1 $\mu\text{g}/\text{mL}$ anti-neurofilament antibody in PBS for 1 hr at RT. After the incubation, excess antibody was washed away with BS, and the slurries were added to the serum containing the Cy5-labeled neurofilament protein. The amount of slurry used in all experiments was normalized to their neurofilament heavy chain binding capacity. Typically, 5–10-fold greater volume of protein A glass bubble slurry was required over that of protein A Sepharose and Dynabeads protein A to obtain equal binding capacity (Figure 2). All serum samples were incubated for 1 hr at RT followed by three washing steps of 1 mL PBS containing 0.1% Tween-20 for 30 min with end-over-end mixing. Proteins were then eluted with 250 μL of 75% ethanol with end-over-end mixing for 30 min at RT. Alternatively, the glass bubbles were destroyed with three 1 sec ultrasonication pulses in 250 μL of 75% ethanol. Total protein in eluted fractions from resins and glass bubbles was determined by bicinchoninic acid (BCA) (Pierce). Empty polypropylene tubes containing only serum were treated in the same way as the slurries, and the protein concentration eluted from the empty tubes was subtracted from protein eluted from the resins and glass bubbles (Figure 3). Samples of the concentrated proteins were also analyzed by SDS-PAGE and stained using a Silver Stain Kit (Pierce).

2.4. Cell Capture Using Glass Microbubbles. The prostate cancer cell line PC-3 (ATCC) was grown to confluency, and 100 μL of cell supernatant was used at first passage. An r-phycoerythrin- (PE-) labeled antibody against epithelial cell adhesion molecule (EpCAM) (eBioscience) was spiked into the cell suspension at 1.25 $\mu\text{g}/\text{mL}$ and incubated for 1 hr. Cells were then suspended in 1 mL of low IgG-FBS and protein A-conjugated glass bubble slurry and an FITC-labeled pan-cytokeratin antibody (Miltenyi Biotech) at 5 $\mu\text{g}/\text{mL}$ was added. Slurry was incubated for 1 hr at RT with end-over-end mixing followed by three washing steps of 1 mL PBS containing 0.1% Tween-20. The bubbles were then mounted overnight on a microscope slide with a fluoromount/4',6-diamidino-2-phenylindole (DAPI) solution (Southern Biotech), which stains DNA, and imaged on a 40X magnification on a fluorescent microscope (Olympus).

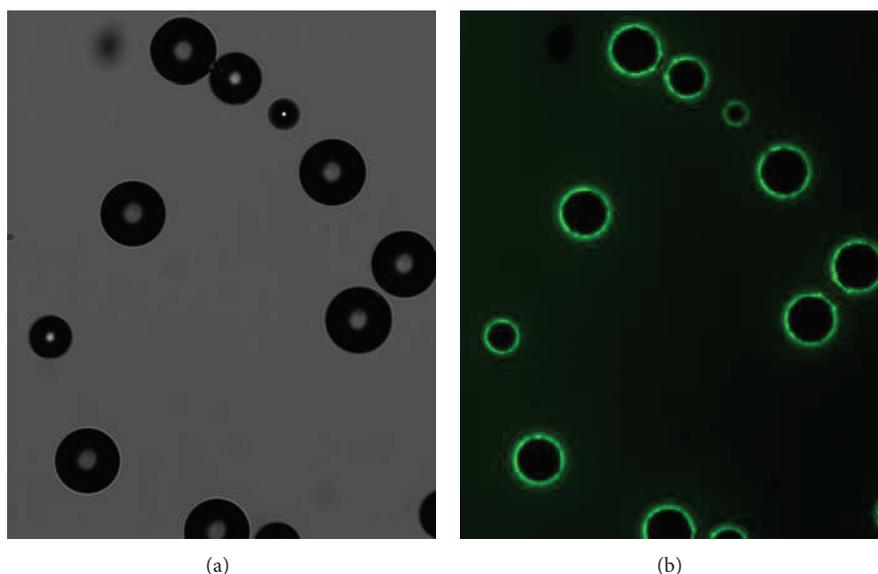


FIGURE 1: Neutravidin conjugated glass microbubbles with bound biotin-fluorescein. Panel A shows a 40X light field of the glass bubbles and panel B shows the surface bound biotin-fluorescein.

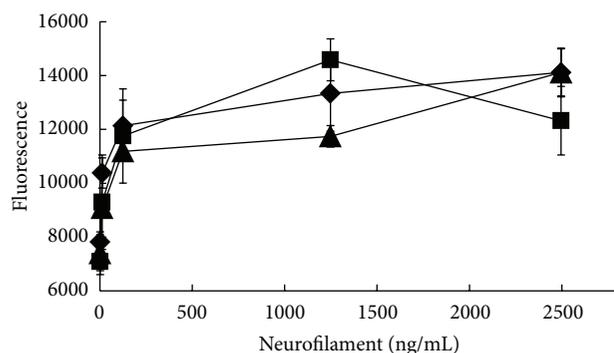


FIGURE 2: Normalization of Protein A Resin and Glass Bubble Binding Capacities. Increasing concentrations of Cy5 labeled neurofilament heavy chain was added to 1 ml aliquots of low-IgG FBS as described in Materials and Methods. Slurries of protein A Sepharose (20 μ L), Dynabeads protein A (10 μ L) or glass bubbles conjugated with protein A (100 μ L) and saturated with anti neurofilament antibodies were added to the tubes and processed as described in Materials and Methods. Closed squares (■) show the protein A glass bubbles, closed diamonds (◆) show the protein A Sepharose and closed triangles (▲) show the protein A Dynabeads.

2.5. Fluorescent Assays. Fluorescent assays were performed using the ultrasensitive Signalyte-II fluorometer (Creatv Microtech, MD) described in more detail in [16–18]. The fluorometer used for these studies was equipped with 4 light emitting diodes (LED) with excitation maxima of 470, 530, 590, and 635 nm and long pass emission filters with cut-on wavelengths of 515, 570, 630, and 665 nm, respectively. The fluorometer cuvettes are glass capillary PCR tubes that can hold a sample size of 20–50 μ L. All fluorescent binding assays were performed in PBS unless otherwise stated. Fluorescence measurements of Cy5-labeled neurofilament heavy chain

were performed with an excitation wavelength of 635 nm, and samples were measured from 100 msec. to 3 sec. per tube.

3. Results and Discussion

Figure 1 shows glass bubbles conjugated with Neutravidin and loaded with fluorescein-labeled biotin. The transparency of the glass makes it possible to observe the uniform fluorescence over the entire surface. To examine the potential of using glass bubbles to isolate low abundance proteins, we used protein A-conjugated glass bubbles to isolate fluorescent neurofilament heavy chain spiked into serum.

The glass microbubbles are very robust and remain intact in batch chromatography with end-over-end mixing, which allows for optimal contact of the bubbles with the analyte. Although glass surfaces can be abrasive, the rate of protein leaching off the glass bubbles with end-over-end mixing was similar to that of protein A Sepharose (data not shown). At the end of the purification procedure, the glass bubbles can be easily destroyed with ultrasonication with the glass shards sinking to the bottom.

Figure 2 shows the binding capacity of the protein A resins and glass bubbles for Cy5-labeled neurofilament heavy chain spiked into serum at various concentrations. Immun isolation of the labeled protein was performed using glass bubbles, Dynabeads, and Sepharose protein A resins. Removing the serum and PBS washes from the tubes containing glass bubbles was in many respects easier than the resins since no centrifugation was required. Additionally, when a pipette tip was used to aspirate liquid from the bottom of the tube, the glass bubbles clung to the side of the tubes. This probably led to more efficient washing steps as residual liquid associated with the glass bubbles drained to the bottom of the tube.

Cy5-labeled neurofilament heavy chain was eluted from the glass bubbles and protein A resins and fluorescence

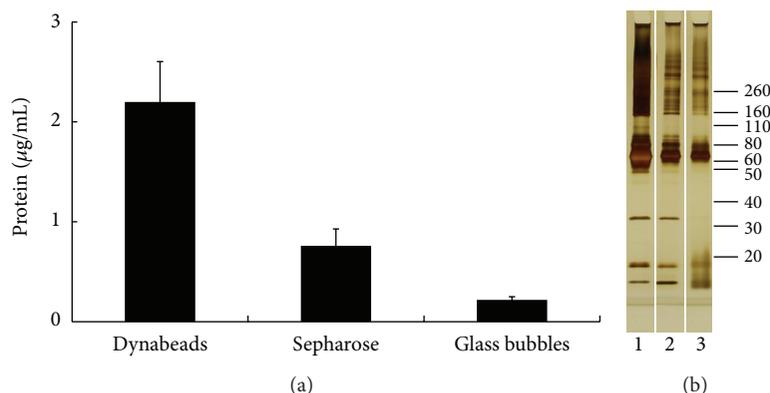


FIGURE 3: Nonspecific serum protein binding to Dynabeads, Sepharose and glass bubbles. Slurries with equivalent neurofilament heavy chain binding capacities were added to serum and processed as described in the Materials and Methods. Panel A shows the total protein eluted from the resins and glass bubbles and panel B shows the protein profile of eluted proteins by SDS-PAGE. Lanes 1, 2 and 3 are proteins eluted from protein A Dynabeads, Sepharose and glass bubbles, respectively. Each lane was loaded with 100 ng of eluted protein.

determined as described in Section 2. Figure 2 demonstrates that to achieve equal binding capacity, approximately 5- to 10-fold greater volume of glass bubble slurry had to be used to equal the binding capacity of the resins, which is consistent with the glass bubbles having a much lower surface area available for immobilizing protein A.

The glass bubbles and resins were washed 3 times using PBS-0.1% Tween-20, which is a rather low-stringency washing condition. Figure 3(a) shows the total protein eluted with 75% ethanol and demonstrates that despite the increased volume of glass bubbles used, the amount of total protein eluted from the glass bubbles was lower compared to the resins. The profiles of the eluted proteins were visualized by SDS-PAGE followed by silver staining. Since most SDS-PAGE protein silver staining is more qualitative than quantitative [19], Figure 3(b) demonstrates that glass bubbles have fewer contaminant species than the Sepharose or Dynabeads resins. However, many protein bands appear to be common for all three. Other researchers have also made similar observations, and Trinkle-Mulcahy et al. [20] developed a methodology to exclude proteins that copurify with the most commonly used resins (Sepharose, agarose, and magnetic beads). Their studies identified over 200 proteins, collectively termed “bead proteome”, as they are specifically associated with the resins. Their results also showed that none of the three resins was superior in terms of reducing background binding. For cytoplasmic extracts, the highest background levels were obtained with magnetic beads [21], and our results show that magnetic beads also had the highest background levels for serum (Figure 3).

The major protein contaminant for the glass bubbles and resins in Figure 3(b) is likely serum albumin, based on the molecular weight. Interestingly, some of these nonspecific proteins in the gel were observed to come from the polypropylene tubes used in the batch absorption process (data not shown).

In addition to isolating proteins from serum, these glass bubbles can also be used to isolate cells from biological samples. The advantage of using the glass bubbles for this task

is that they remain buoyant even when they are centrifuged. Cells that are not bound to the bubbles are pelleted and thus easily separated. Furthermore, cells need not be separated from the bubbles for imaging as they are transparent. Figure 4 shows a representative sample of PC-3 prostate carcinoma cells captured by protein A-conjugated glass bubbles. Whole cultured cells were first incubated with PE-labeled EpCAM antibody, which also served as the capture antibody for the protein A microbubbles. Only cells with EpCAM surface antigen were captured while the remaining non-EpCAM cells were removed from the population. Cells stained with EpCAM (in red) show a typical cell surface staining pattern. The potential benefits of using transparent glass bubbles for capturing cells can be seen in panels 1(a)–1(e) where a cell that is not directly in the field of vision can be observed through the glass.

The PC-3 cells captured on glass bubbles, visible light (row (a)), were also stained with FITC-labeled cytokeratin antibody (row (b)). The cytokeratin antibody (in green) shows a filamented cytoplasmic staining, EpCAM antibody labeled red (row (c)). Cells were then mounted with a DAPI mounting solution, which stains the nucleus (row (d)). Finally, row (e) is the merged images of the cells. An additional advantage of using the glass bubbles was the low fluorescent background, which could be due to their low nonspecific protein binding. This allowed for long exposures and high quality images of the staining patterns of cells isolated using glass bubbles.

4. Conclusions

Previously, our group and others have examined the utility of using buoyant glass microbubbles to capture cells [21, 22]. The advantage of using glass bubbles to capture cells is that they are easily separated from liquid and their transparency is a benefit if captured cells are stained with fluorescent markers. Circulating tumor cells (CTCs) are an example where high purity cell isolation from a complex mixture may benefit from glass bubble chromatography. In this study, we expand

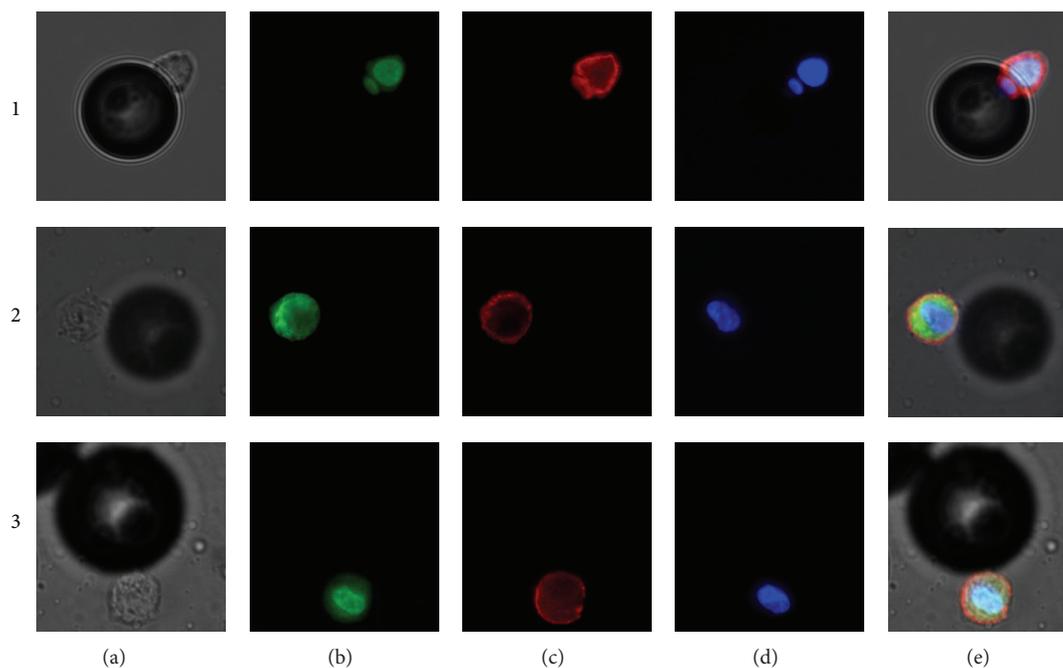


FIGURE 4: PC-3 cells binding to protein A conjugated glass microbubbles. Prostate cancer cells (PC-3), with PE labeled EpCAM, bound to protein A glass microbubbles (row A in visible light) were stained with FITC labeled anti cytokeratin (row B), PE labeled anti EpCAM (row C) and DAPI (row D). Row E shows merged images of the stained cells (40X magnification).

the scope of glass bubble chromatography to include low abundance proteins. By their very nature, glass bubbles are inferior to conventional chromatography resins with respect to binding capacity and flow characteristics. However, in certain applications where the purity of an isolated protein is more important than the yield, glass microbubbles could be a useful alternative to traditional resins.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgment

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References

- [1] J. Margolis, "Activation of plasma by contact with glass: evidence for a common reaction which releases plasma kinin and initiates coagulation," *The Journal of Physiology*, vol. 144, no. 1, pp. 1–22, 1958.
- [2] D. W. Barnes and J. Silnutzer, "Isolation of human serum spreading factor," *Journal of Biological Chemistry*, vol. 258, no. 20, pp. 12548–12552, 1983.
- [3] E. G. Hayman, M. D. Pierschbacher, Y. Ohgren, and E. Ruoslahti, "Serum spreading factor (vitronectin) is present at the cell surface and in tissues," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 80, no. 13, pp. 4003–4007, 1983.
- [4] P. Cuatrecasas, "Affinity chromatography," *Annual Review of Biochemistry*, vol. 40, pp. 259–278, 1971.
- [5] S. K. Bhatia, L. C. Shriver-Lake, K. J. Prior et al., "Use of thiol-terminal silanes and heterobifunctional crosslinkers for immobilization of antibodies on silica surfaces," *Analytical Biochemistry*, vol. 178, no. 2, pp. 408–413, 1989.
- [6] J. N. Adkins, S. M. Varnum, K. J. Auberry et al., "Toward a human blood serum proteome: analysis by multidimensional separation coupled with mass spectrometry," *Molecular & Cellular Proteomics*, vol. 1, no. 12, pp. 947–955, 2002.
- [7] N. L. Anderson and N. G. Anderson, "The human plasma proteome: history, character, and diagnostic prospects," *Molecular & Cellular Proteomics*, vol. 1, no. 11, pp. 845–867, 2002.
- [8] N. Tang, P. Tornatore, and S. R. Weinberger, "Current developments in SELDI affinity technology," *Mass Spectrometry Reviews*, vol. 23, no. 1, pp. 34–44, 2004.
- [9] Q. Liu, F. Xie, A. Alvarado-Diaz et al., "Neurofilamentopathy in neurodegenerative diseases," *Open Neurology Journal*, vol. 5, no. 1, pp. 58–62, 2011.
- [10] D. M. Toivola, P. Strnad, A. Habtezion, and M. B. Omary, "Intermediate filaments take the heat as stress proteins," *Trends in Cell Biology*, vol. 20, no. 2, pp. 79–91, 2010.
- [11] N. Norgren, L. Rosengren, and T. Stigbrand, "Elevated neurofilament levels in neurological diseases," *Brain Research*, vol. 987, no. 1, pp. 25–31, 2003.
- [12] J. Brettschneider, A. Petzold, S. D. Süßmuth et al., "Neurofilament heavy-chain NfHSMI35 in cerebrospinal fluid supports the differential diagnosis of parkinsonian syndromes," *Movement Disorders*, vol. 21, no. 12, pp. 2224–2227, 2006.

- [13] J. Brettschneider, A. Petzold, D. Schöttle, A. Claus, M. Riepe, and H. Tumani, "The neurofilament heavy chain (NfH^{SM135}) in the cerebrospinal fluid diagnosis of Alzheimer's disease," *Dementia and Geriatric Cognitive Disorders*, vol. 21, no. 5-6, pp. 291-295, 2006.
- [14] A. Antinori, G. Arendt, J. T. Becker, B. J. Brew, D. A. Byrd, and D. B. Clifford, "Biomarkers of HIV-associated neurocognitive disorders," in *Proceedings of the HIV Infection and the Central Nervous System: Developed and Resource Limited Settings*, Rome, Italy, June 2005.
- [15] M. Gisslén, L. Rosengren, L. Hagberg, S. G. Deeks, and R. W. Price, "Cerebrospinal fluid signs of neuronal damage after antiretroviral treatment interruption in HIV-1 infection," *AIDS Research and Therapy*, vol. 2, no. 1, article 6, 2005.
- [16] P. Zhu, D. R. Shelton, S. Li et al., "Detection of *E. coli* O157:H7 by immunomagnetic separation coupled with fluorescence immunoassay," *Biosensors and Bioelectronics*, vol. 30, no. 1, pp. 337-341, 2011.
- [17] S. Li, Y. Zhang, P. Amstutz III, and C.-M. Tang, "Multiplex integrating waveguide sensor: signalite-II," *Methods in Molecular Biology*, vol. 503, pp. 423-434, 2009.
- [18] S. Li, L. Kiefer, P. Zhu et al., "Ultra-sensitive detection using integrated waveguide technologies," in *Biosensors For Health, Environment and Biosecurity/Book 3*, Intech, Rijeka, Croatia, 2011.
- [19] H. M. Poehling and V. Neuhoff, "Visualization of proteins with a silver "stain": a critical analysis," *Electrophoresis*, vol. 2, pp. 141-147, 1981.
- [20] L. Trinkle-Mulcahy, S. Boulon, Y. W. Lam et al., "Identifying specific protein interaction partners using quantitative mass spectrometry and bead proteomes," *Journal of Cell Biology*, vol. 183, no. 2, pp. 223-239, 2008.
- [21] S. Stefansson, D. Adams, and C. M. Tang, "Sample preparation using buoyant silica microspheres," in *Proceedings of the 6th Early Detection Research Network Workshop (EDRN '09)*, Bethesda, Md, USA, August-September 2009.
- [22] C. H. Hsu, C. C. Chen, D. Irimia, and M. Toner, "Isolating cells from blood using Buoyancy Activated Cell Sorting (BACS) with glass microbubbles," in *Proceedings of the 14th International Conference on Miniaturized Systems for Chemistry and Life Sciences*, Groningen, The Netherlands, October 2010.



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