The binding of avidin to biotin is the strongest known noncovalent biological interaction [dissociation constants ($K_d$) approximately $10^{-15}$ M] between a protein and ligand (1). The high affinity and specificity of the avidin-biotin interaction is utilized for enriching biotin-containing proteins from mixtures (2,3). Water-soluble N-hydroxysulfosuccinimide (sulfo-NHS) derivatives of biotin are compatible with biological systems, providing the opportunity to label proteins in biological samples with biotin as a component of purification protocols. Sarbarth et al. (4), for example, used the biotinylation of Helicobacter proteins to isolate and identify the proteins expressed at the cell surface. Immobilized tetrameric avidin was used to capture the biotinylated proteins, and proteins were eluted using 8 M guanidine HCl at pH 1.5. More recently, immobilized monomeric avidin with a lower binding affinity for biotin ($K_d$ approximately $10^{-8}$ M) has become commercially available. This reagent permits recovery of biotinylated proteins and peptides using milder elution conditions, thus preserving native protein conformation and enzyme activity (5).

In experiments to biotinylate proteins using biotin conjugates with different spacer arm lengths, we have observed differential protein recovery when immobilized polymeric or monomeric avidin is used. Our results illustrate the importance of selecting the optimal combination of biotinylation and avidin reagents for the recovery of the proteins of interest in proteomics studies.

To biotinylate the outer membrane proteins of *Shewanella oneidensis* MR-1, 1 g (wet weight) of cells were harvested and biotinylated as described by Sabarth et al. (4) using sulfo-succinimidyl-6-(biotinamido)-6-hexanamido hexanoate (EZ-Link® Sulfo-NHS-LC-Biotin; Pierce Chemical, Rockford, IL, USA), sulfo-succinimidyl-6'- (biotinamido)-6-hexamodo hexanoate (EZ-Link Sulfo-NHS-LC-LC-Biotin; Pierce Chemical) or a 50:50 combination of the two reagents. After biotinylation, cells were sonicated, and the membrane fraction was isolated using differential centrifugation (9000 × g for 10 min at 4°C to remove unbroken cells; 40,000 × g for 30 min at 4°C to recover the membranes). Membranes were suspended in 500 μL 50 mM Tris-HCl, 150 mM KCl, and 10 mM EDTA, pH 7.4. Biotinylated proteins were released by adding 1 mL 2% Zwittergent® 3-14 (Calbiochem, San Diego, CA, USA) and incubating for 1–2 h at 4°C. Soluble biotinylated proteins were collected in supernatants after ultracentrifugation at 100,000 × g for 1 h at 4°C.

For polyvalent avidin capture of biotinylated proteins, supernatants diluted 1:5 with 100 mM NaPO₄, 150 mM NaCl, pH 7.2 [phosphate-buffered saline (PBS)] were loaded onto Immunopure® AffinityPak™ Immobilized Avidin columns (Pierce Chemical) equilibrated with PBS plus 0.2% Zwittergent. After incubation for 30 min at room temperature, the columns were washed five times with PBS plus 0.2% Zwittergent. Biotinylated proteins were eluted at 37°C with five column volumes of PBS plus 0.2% Zwittergent containing 5 mM D-biotin, followed by five column volumes of 8 M guanidine hydrochloride buffer, pH 1.5, at room temperature. Combined protein eluates were concentrated by acetone precipitation.

![Figure 1. *Shewanella oneidensis* proteins captured by polymeric avidin after biotinylation.](image-url)

Intact cells grown under aerobic conditions in defined media were labeled with (A) EZ-Link Sulfo-NHS-LC-Biotin, (B) EZ-Link Sulfo-NHS-LC-LC-Biotin, or (C) a 50:50 mixture of EZ-Link Sulfo-NHS-LC-Biotin and EZ-Link Sulfo-NHS-LC-LC-Biotin. Proteins were separated by two-dimensional gel electrophoresis (2-DE) using a 2:1 mixture of pH 5.0–7.0 and pH 3.0–10.0 carrier ampholytes for the isoelectric focusing dimension (6) and a linear gradient of 10%–17% polyacrylamide for the second dimension (7). Proteins were detected by Western blot analysis using neutravidin. Patterns are oriented with acidic proteins to the left, basic proteins to the right, high molecular weight proteins toward the top, and low molecular weight proteins toward the bottom. The isoforms (chains of spots) visible on the 2-DE patterns are due to the charge-modifications generated by the binding of biotin molecules to the amino groups of lysine residues and the amino terminus of each protein. pl, isoelectric point.
Alternatively, biotinylated proteins were captured using Immunopure Immobilized Monomeric Avidin Gel (Pierce Chemical). After blocking the irreversible biotin binding sites, columns were loaded with biotinylated protein samples diluted 1:5 and incubated for 1 h at room temperature. Columns were washed six times with PBS containing 0.2% Zwittergent. Biotinylated proteins were eluted with 10 column volumes of PBS containing 0.2% Zwittergent and 5 mM D-biotin, and combined protein eluates were concentrated by acetone precipitation. Acetone-precipitated proteins were solubilized in 100 μL 9 M urea, 4% Nonidet™ P40 (NP40), 2% mercaptoethanol, and 2% ampholyte, pH 8.0–10.0 (Bio-Rad Laboratories, Hercules, CA, USA). Five-microliter aliquots were separated by two-dimensional gel electrophoresis (2-DE) (6,7). After 2-DE, proteins were transferred to Hybond®-C membranes (Amersham Biosciences, Piscataway, NJ, USA) by semi-dry electroblotting. The membranes were then incubated for 30 min with 20 mM Tris-HCl, 140 mM NaCl, and 5 mM KCl, pH 7.0, containing 0.05% Tween® 20, followed by incubation with horseradish peroxidase-conjugated neutravidin (1:400 dilution) for 1 h at room temperature. Reactive proteins were detected by chemiluminescence with SuperSignal® West Femto maximum sensitivity substrate (Pierce Chemical). Blots were exposed to X-ray film for 1, 5, and 10 min to generate images that revealed the full range of proteins from high to low abundance. The X-ray film images were digitized using an Eikonix 1412 charge-coupled device scanner interfaced with a VAX 4000-90 workstation (Hewlett-Packard, Loveland, CO, USA).

When polymeric avidin was used to capture the *S. oneidensis* proteins labeled with either Sulfo-NHS-LC-biotin or Sulfo-NHS-LC-LC-biotin, similar proteins were detected with varying relative chemiluminescence intensity for some proteins when patterns from the two reagents were compared (Figure 1, A and B). Cells labeled with a 50:50 mixture of the two biotinylation reagents yielded significantly more protein spots than detected when either reagent was used alone (Figure 1C). The relative abundance of proteins common to all three labeling protocols is comparable, indicating that the additional proteins detected when both reagents were used for biotinylation were additional reaction products, not the result of different times of exposure to the X-ray film. These results were reproducible in triplicate experimental replicates.

2-DE revealed a dramatic increase in the number of proteins captured when monovalent avidin was used to capture biotinylated proteins, regardless of the labeling reagent used (Figure 2). The range of X-ray exposures used consistently revealed capture of a more complex mixture of proteins by the monomeric avidin than the polymeric avidin. In addition, the proteins captured from samples labeled with the 50:50 mixture of biotinylation reagents were a combination of those found in samples labeled with one reagent or the other; no new proteins were observed, in contrast to when the polymeric avidin was used.

The additional complexity of the proteins recovered using the monomeric avidin suggests that use of the lower affinity avidin results in more efficient recovery of biotinylated proteins than obtained using the higher affinity polymeric avidin. Since the labeled proteins were detected using neutravidin, only the biotinylated proteins bound and released from the monovalent avidin are shown in the 2-DE patterns. Thus, nonspecific binding of nonbiotin proteins, which could occur due to the lower affinity of the monovalent avidin, did not influence the number of biotinylated proteins detected. The specificity of both the polyvalent and monovalent avidin matrices was further verified by the identification of proteins detected in the 2-DE patterns. Since the labeling reaction was performed with intact cells, the most abundant biotinylated proteins (i.e., the most intensely chemiluminescent in the neutravidin detection protocol) were expected to be outer membrane proteins. In preliminary studies to identify cell surface proteins captured using the

![Figure 2. *Shewanella oneidensis* proteins captured by low affinity monomeric avidin after biotinylation.](image-url)
biotinylation tagging method, a putative outer membrane porin (gi24375384), a tetratricopeptide repeat (TPR) domain protein (gi24373394), and a putative major outer membrane lipoprotein (gi24372875) have been consistently found as major components in samples from experiments with either or both biotinylation labels using either polyvalent or monovalent avidin for protein capture. A putative membrane protein (gi24375951) captured by the monovalent but not the polyvalent avidin (one of the proteins in the lower left corner of all of the patterns shown in Figure 2) has also been identified.

The recovery of more proteins from samples labeled with a mixture of both biotinylation reagents instead of either reagent alone when polyvalent avidin was used suggests that proteins labeled with both long- and short-armed labeling reagents had reduced binding affinity and were, therefore, released more efficiently from the tetrameric avidin compared with proteins labeled with one reagent or the other. The reason for this reduced binding affinity is unknown, but could be due to steric hindrance introduced in proteins labeled with more than one type of reagent.

The results from these experiments demonstrate that researchers should be aware of the advantages and disadvantages of the various biotin and avidin derivatives available, as markedly different results can be obtained with each of them. The differences observed in the neutravidin-stained 2-DE images of S. oneidensis proteins in our study were due to the differential elution of proteins from polymeric and monomeric avidin gels used for affinity purification. So for this particular study, the selection of avidin for the purification of biontilylated proteins was the most critical step in the labeling and purification procedure. For our studies of S. oneidensis surface membrane proteins, the monomeric avidin is providing the best alternative for capturing the maximum number of labeled proteins.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES


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Address correspondence to Carol S. Giometti, Argonne National Laboratory, Biosciences Division, 9700 South Cass Avenue, Argonne, IL 60439, USA. e-mail: csgiometti@anl.gov

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