4.1 Introduction to Avidin–Biotin and Antibody–Hapten Techniques

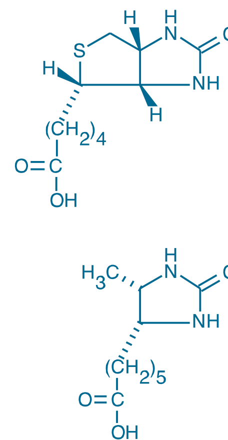

Figure 4.1 Comparison of the structures of D-biotin (top) and D-desthiobiotin (bottom).

The high affinity and specificity of avidin–biotin and antibody–hapten interactions have been exploited for diverse applications in immunology, histochemistry, *in situ* hybridization (Section 8.5), affinity chromatography and many other areas.[1–3] Biotinylation (Table 4.1) and haptenylation (Table 4.2) reagents provide the "tag" that transforms poorly detectable molecules into probes that can be recognized by a labeled detection reagent or an affinity-capture matrix. Once tagged with biotin or a hapten, a molecule of interest — such as an antibody, lectin, drug, polynucleotide, polysaccharide or receptor ligand — can be used to probe complex solutions, cells and tissues, as well as protein and nucleic acid blots and arrays. This tagged molecule can then be detected with the appropriate avidin or anti-hapten antibody conjugate that has been labeled with a fluorophore, fluorescent microsphere (Section 6.5), enzyme, chromophore, magnetic particle or colloidal gold (Section 7.6). Biotinylated molecules can also be captured with various forms of immobilized streptavidin, such as our streptavidin agarose (S-951, Section 7.6), CaptAvidin agarose (C-21386, Section 7.6) and Captivate ferrofluid magnetic particles (C-21476, Section 7.6), or stained for electron microscopy with NANOGOLD or Alexa Fluor FluoroNanogold streptavidin 1.4 nm gold clusters (N-24918, A-24926, A-24927, Section 7.6).

Although binding of biotin to native avidin or streptavidin is essentially irreversible, appropriately modified avidins can bind biotinylated probes reversibly, making them valuable reagents for isolating and purifying biotinylated molecules from complex mixtures.[4,5] CaptAvidin biotin-binding protein (C-21385, Section 7.6) is our newest avidin derivative.[5] Selective nitration of tyrosine residues in the four biotin-binding sites of avidin considerably reduces the affinity of the protein for biotinylated molecules above pH 9. Consequently, biotinylated probes can be adsorbed at neutral pH or below and released at ~pH 10[4,5] (Figure 7.85). CaptAvidin agarose (C-21386, Section 7.6) is particularly useful for separating and purifying biotin conjugates from complex mixtures.[5]

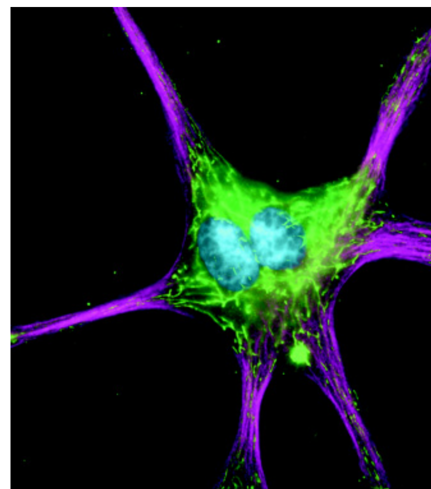

Figure 4.2 The cytoskeleton of a fixed and permeabilized bovine pulmonary artery endothelial cell detected using mouse monoclonal anti–α-tubulin antibody (A-11126), visualized with Alexa Fluor 647 goat anti–mouse IgG antibody (A-21235) and pseudocolored magenta. Endogenous biotin in the mitochondria was labeled with green-fluorescent Alexa Fluor 488 streptavidin (S-11223) and DNA was stained with blue-fluorescent DAPI (D-1306, D-3571, D-21490).

In contrast to the modified avidin of our CaptAvidin products, our unique DSB-X biotin technology (Section 7.6) employs a modified biotin to provide a means of labeling and separating biomolecules, including live cells, under extremely gentle conditions. The DSB-X biotin reagents, which are derivatives of desthiobiotin (Figure 4.1) with an additional seven-atom 'X' spacer, have moderate affinity for avidin and streptavidin that is rapidly reversed by low concentrations of free biotin or desthiobiotin at neutral pH and room temperature (Figure 7.89). This technique permits capture and release of DSB-X biotin–labeled molecules using our DSB-X Biotin Bioconjugate Isolation Kits #1 and #2 (D-20658, D-20659; Section 7.6; Figure 7.95) or Captivate ferrofluid streptavidin (C-21476, Section 7.6).

Avidin–biotin and antibody–hapten techniques are compatible with flow cytometry and light, electron and fluorescence microscopy, as well as with solution-based methods such as enzyme-linked immunosorbent assays (ELISAs). Moreover, avidin–biotin and antibody–hapten techniques are frequently combined for simultaneous, multicolor detection of multiple targets in a complex solution, cell or tissue sample. Furthermore, by judicious choice of detection reagents and sandwich protocols, these techniques can be employed to amplify the signal from low-abundance analytes. For example, the bridging method is a common immunohistochemical technique for signal amplification and improved tissue penetration in which avidin or streptavidin serves as a bridge between two biotinylated molecules.

Our exclusive DSB-X biotin technology (Section 4.3) retains all the advantages of biotinylated probes for affinity labeling but its binding can be readily reversed at neutral pH in common buffers.

This chapter is devoted to our biotinylation, desthiobiotinylation (DSB-X biotinylation) and haptenylation reagents (Section 4.2) and our biotin and desthiobiotin (DSB-X biotin) conjugates (Section 4.3). Section 7.3 and Section 7.6 describe our large assortment of labeled antibody (Table 7.3) and avidin (Table 7.17) probes; we offer the largest selection of dyes available from any commercial source. Our unique Zenon One Mouse IgG_1 Labeling Kits (Section 7.2) permit the rapid and quantitative labeling of even extremely small quantities of mouse monoclonal antibodies with biotin, DSB-X biotin or a wide variety of fluorophores and enzymes (Section 7.2, Table 7.1, Figure 7.32). Where they can be used, our avidin- and biotin-coated FluoSpheres and TransFluoSpheres fluorescent microspheres (Section 6.5) provide an alternative detection technology that offers

a combination of fluorescence intensity and photostability far superior to that of any simple dye conjugate.

Both the tyramide signal-amplification (TSA) technology (Section 6.2), which was developed by NEN (now a part of PerkinElmer Corporation) and licensed to Molecular Probes for in-cell and in-tissue applications, and our Enzyme-Labeled Fluorescence (ELF) technology (Section 6.3) take advantage of the high affinity and specificity of the avidin–biotin interaction. Several of our ELF 97 kits utilize an application-specific ELF 97 phosphatase substrate and either a streptavidin–alkaline phosphatase conjugate or a biotinylated alkaline phosphatase plus a streptavidin bridge to yield much greater fluorescence at the site of biotinylated molecules than is possible with direct dye conjugates of avidin. Additionally, Molecular Probes has introduced several TSA kits (Section 6.2, Table 6.1) that contain a labeled tyramide and a horseradish peroxidase conjugate of either streptavidin or a secondary antibody for amplifying the detectability of biotinylated or antibody-labeled molecules (Figure 6.6). Six of our TSA kits utilize either biotin-XX tyramide or DSB-X biotin as the amplification reagent and three of the TSA kits use the 2,4-dinitrophenyl (DNP) group as a hapten. Our 27 other TSA kits utilize one of our Alexa Fluor tyramides, Pacific Blue tyramide or Oregon Green 488 tyramide as the direct detection reagents. Our antibodies to the Alexa Fluor 488 and Oregon Green dyes (Section 7.4, Table 7.13) permit these dyes to be used for either direct detection or further amplification schemes. Further amplification can sometimes be achieved by combining TSA with ELF or a second round of TSA (Figure 6.6) to yield the greatest fluorescence intensity in cells and tissues, while retaining high spatial resolution.

Mammalian cells and tissues contain biotin-dependent carboxylases, which are required for a variety of metabolic functions. These biotin-containing enzymes often produce substantial background signals when biotin–avidin or biotin–streptavidin detection systems are used to identify cellular targets[6] (Figure 4.2, Figure 12.28). The reagents in our Endogenous Biotin-Blocking Kit (E-21390), which is described in Section 7.6, can be used to minimize interference from endogenous biotin in these techniques.

References

1. Meth Enzymol 184, (Complete Volume) (1990); **2.** Anal Biochem 171, 1 (1988); **3.** Methods Biochem Anal 26, 1 (1980); **4.** Biochem J 316, 193 (1996); **5.** Anal Biochem 243, 257 (1996); **6.** J Histochem Cytochem 45, 1053 (1997).

4.2 Biotinylation and Haptenylation Reagents

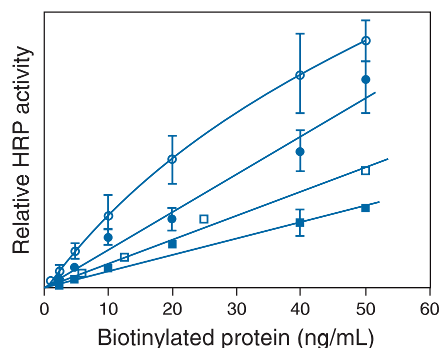

Figure 4.3 ELISA-type assay comparing the binding capacity of bovine serum albumin (BSA) and goat anti–mouse IgG antibody (GAM) biotinylated with either biotin-X or biotin-XX. The assay was developed using a streptavidin–horseradish peroxidase conjugate (S-911, 0.2 µg/mL) and *o*-phenylenediamine dihydrochloride (OPD). The moles of biotin per mole of protein were: 4.0 biotin-X/GAM (●), 4.4 biotin-XX/GAM (○), 6.7 biotin-X/BSA (■) and 6.2 biotin-XX/GAM (□). Error bars on some data points have been omitted for clarity. Reprinted with permission from Methods Mol Biol 45, 223 (1995).

Our numerous avidin and streptavidin products are described in Section 7.6.

Molecular Probes is the primary manufacturer of a diverse array of biotinylation (Table 4.1) and haptenylation (Table 4.2) reagents for labeling biomolecules. In addition, our DSB-X biotin technology (Section 7.6) employs a modified biotin to provide a means of labeling and separating biomolecules, including live cells, under extremely gentle conditions.[1] Biotinylated molecules, as well as DSB-X biotin–labeled molecules prepared using our DSB-X Biotin Protein Labeling Kit (D-20655, Section 1.2), can be subsequently detected with fluorescent dye or enzyme conjugates of avidins (Section 7.6, Table 7.17) or with NANOGOLD streptavidin gold clusters (N-24918, A-24925, A-24926; Section 7.6). These biotinylated and DSB-X biotin–labeled molecules are also readily captured and separated from solutions with streptavidin agarose (S-951, Section 7.6, Figure 7.89), CaptAvidin agarose (C-21386, Section 7.6) or the streptavidin conjugate of Captivate ferrofluid superparamagnetic particles (C-21476, Section 7.6). Adsorption of DSB-X biotin–labeled molecules or cells onto affinity matrices can be rapidly reversed at neutral pH and room temperature by adding free biotin to the solution (Figure 7.85).

Reviews of the methods that we use to prepare biotin[2,3] and fluorescent[4] conjugates of antibodies have been published. To make the labeling reactions particularly easy, we have developed some very useful kits for labeling proteins with biotin, DSB-X biotin, 2,4-dinitrophenyl (DNP) or a choice of several different fluorophores; see Section 1.2 for a complete description of these products. Each of the protein labeling kits contains the preferred reactive dye or hapten — many of which have spacers to reduce interactions between the label and the biomolecule — along with a detailed protocol for preparing the conjugates. In most cases, these kits also provide the separation media for purifying labeled protein conjugates from the reaction mixture.

Our ARES DNA Labeling Kits (Section 8.2, Table 8.8, Figure 8.44), ULYSIS Nucleic Acid Labeling Kits (Section 8.2, Table 8.7, Figure 8.41), Alexa Fluor Oligonucleotide Amine Labeling Kits (Section 8.2, Table 8.9) and ChromaTide UTP, dUTP and dCTP nucleotides (Section 8.2; Table 8.5, Table 8.6), yield probes whose labels can, in many