

The Biotin–(Strept)Avidin System: Principles and Applications in Biotechnology

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The biotin–(strept)avidin system has been used for many years in a variety of different applications. Here we present a general overview of the system, describe its components and advantages, and show how the system is used in various applications, with emphasis on immunological and nucleic acid hybridization assays. This system is now considered a versatile independent technology with broad applications in many branches of biotechnology. Clearly, its use will continue to grow in the years to come.

Additional Keyphrases: *nonisotopic immunoassay · nucleic acid hybridization · affinity chromatography · labeling techniques · DNA probes*

Living organisms usually develop highly specific defense mechanisms, which help them survive in competitive and unfriendly environments. Scientists sometimes unravel such defense mechanisms and use them *in vitro*, in a completely different context. For example, bacterial restriction endonuclease enzymes revolutionized the field of molecular cloning when molecular biologists recognized in them a cheap, easy, and highly specific and versatile way of cutting nucleic acids. Here we will discuss the utility of avidin, a protein found in egg white, and streptavidin, a similar protein found in *Streptomyces avidinii*, which have the ability to bind with very high affinity the vitamin biotin (1). This interaction is thought to represent a natural defense mechanism because the binding with avidin or streptavidin of biotinylated enzymes that participate in CO₂ transfer inactivates the enzymes and thus inhibits the growth of bacteria that depend on biotinylated enzymes.

Although many other ligand–binder interactions are described in the literature (2), the biotin–avidin or biotin–streptavidin interaction has some unique characteristics that make it ideal as a general bridge system in many diverse applications:

(a) The noncovalent interaction of avidin or streptavidin with biotin is characterized by a formation (affinity) constant of 10¹⁵ L · mol⁻¹. This is among the highest formation constants reported, about 10³–10⁶ times greater than for the interaction of ligands with

their specific antibodies. This high affinity ensures that, once formed, the complex is not disturbed by changes in pH, the presence of chaotropes, or manipulations such as multiple washings when the complex is immobilized.

(b) Avidin or streptavidin binding to biotin is specific enough to ensure that the binding is directed only to the target of interest.

(c) Both streptavidin and avidin possess four binding sites per molecule. This very useful property makes it possible to use multiply biotinylated moieties (e.g., polybiotinylated enzymes) and avidin or streptavidin to create mixtures consisting of polymers of biotinylated moieties with avidin or streptavidin (3). These polymers could still have some free binding sites for biotin, thus becoming more-sensitive detection reagents in pertinent applications.

(d) Biotin is a small molecule (244.31 Da) that, when introduced into biologically active macromolecules, in most cases does not affect their biological activity, e.g., enzymic catalysis or antibody binding. Moreover, using biotin to derivatize small molecules (e.g., mononucleotides or thyroid or steroid hormones) yields biotinylated moieties that still can act as enzyme substrates or are able to bind specific antibodies. Thus, biotinylation does not usually alter many properties of the molecules.

(e) On many occasions, avidin or streptavidin must be chemically derivatized with various organic reagents for conjugation with low- or high- *M_r* compounds or solid supports. As a rule, both streptavidin and avidin are exceptionally stable molecules and their biotin-binding activity can survive harsh reaction conditions and extensive derivatizations (4).

In the biotin–avidin or biotin–streptavidin system, one participating component must always be biotinylated (Table 1). The various biotinylation strategies and chemical reactions will be mentioned in detail below. Fortunately, many biotinylation reagents are now commercially available; these are extremely easy to use by following well-established procedures available in the literature and from the reagent manufacturers. Also, highly purified avidin and streptavidin are commercially available and they are relatively cheap. Large collections of derivatized avidin and streptavidin—e.g., with fluorophores, enzymes, metals, proteins, solid phases—are also commercially available (Table 2). These reagents, in combination with biotinylated moieties (Table 1), have contributed in the quick dissemination of diverse applications and encouraged many investigators to use the system.

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Table 1. Selected List of Biotinylated Molecules and Their Possible Applications

Biotinylated moiety	Possible applications
Anti-immunoglobulins, Protein A, Protein G	Immunological assays, flow cytometry, cell sorting, immunohistochemistry, Western blots
Lectins	Glycoconjugate studies, mitogenic stimulation studies
Anti-lectins	Localization of lectin receptors
Enzymes (ALP, β -galactosidase, glucose oxidase, HRP)	Immunological assays, nucleic acid hybridization
Ferritin, hemocyanin	Electron microscopy
Agarose, cellulose	Affinity chromatography
Anti-avidin, anti-streptavidin	Amplification assays
Nucleotides	Nucleic acid hybridization
DNA	Nucleic acid hybridization, molecular mass markers, DNA sequencing
Hormones	Affinity chromatography, receptor-ligand interaction studies
Cells	Hybridoma production

ALP, alkaline phosphatase; HRP, horseradish peroxidase.

Table 2. Selected List of Avidin or Streptavidin Conjugates and Their Possible Applications

Conjugate of avidin or streptavidin with	Possible applications
Enzymes (ALP, HRP, β -galactosidase, glucose oxidase, etc), fluorophores (fluorescein, coumarins, rhodamines, phycoerythrin, Texas Red), Eu^{3+} , other metals	Immunological assays, flow cytometry, cell sorting, immunohistochemistry, Western blots, nucleic acid hybridization
Eu^{3+} -chelates ^a	Immunological assays, Western blots, nucleic acid hybridization
Ferritin, gold	Electron microscopy
Chemiluminescent labels	Immunological assays, Western blots, nucleic acid hybridization
Agarose	Affinity chromatography
Magnetic particles	Nucleic acid hybridization, affinity chromatography, DNA sequencing
Polystyrene	Immunological assays

^a With 4,7-bis-chlorosulfonylphenyl-1,10-phenanthroline-2,9-dicarboxylic acid. Abbreviations as in Table 1.

The literature on the system has exploded during the last 10 years, and many specialized reviews have been published (5-11). A comprehensive volume of *Methods in Enzymology* is devoted to the avidin-biotin technology (12) and can serve as a reference for interested readers and experimentalists. Given the special interests of the readers of *Clinical Chemistry*, we will cover, with some detail, applications of the biotin-avidin system in immunochemical and nucleic acid hybridization techniques. Other applications will also be mentioned.

Avidin and Streptavidin

Avidin, a 67-kDa glycoprotein, consists of four identical subunits of 128 amino acids each. The amino acid sequence of the subunits is known (13). Recently, Gope et al. cloned the avidin gene from chicken oviduct (14). The cloned gene was successfully expressed in *Escherichia coli*, providing biologically active recombinant avidin.

In theory, avidin could be purified by adsorption on an insoluble biotinylated matrix; however, because of the very high affinity of the biotin-avidin complex, the avidin is not easily eluted. Today, affinity-purified avidin can be prepared by using insoluble matrices, e.g., Sepharose covalently linked with iminobiotin (15-19). Iminobiotin is a biotin derivative (Figure 1) containing a guanidinium group instead of a ureido group. When the guanidinium group is not protonated, i.e., is at relatively high pH (e.g., 11.0), iminobiotin binds strongly to avidin, although with ~100-fold less affinity than biotin. At low pH (e.g., 4.0), the guanidinium group is protonated, and iminobiotin does not bind to avidin. Thus, avidin can be specifically adsorbed on an iminobiotin-Sepharose matrix at pH 11.0 and then eluted in pure form at pH 4.0.

Avidin is a glycoprotein containing both mannose and *N*-acetylglucosamine (13) and has an isoelectric point (pI) of ~10 (2). The major problem of using avidin in some applications is the high nonspecific binding, which is attributed to both the presence of the sugars and the high pI. Nonglycosylated avidin has essentially the same biotin-binding characteristics as glycosylated avidin and can be isolated from crude avidin preparations (~30% of total) by using concanavalin-A affinity columns that adsorb only the glycosylated fraction (20, 21). Alternatively, deglycosylation by enzymatic cleavage could be used. Deglycosylated avidins are expected to have lower nonspecific binding. Commercial manufac-

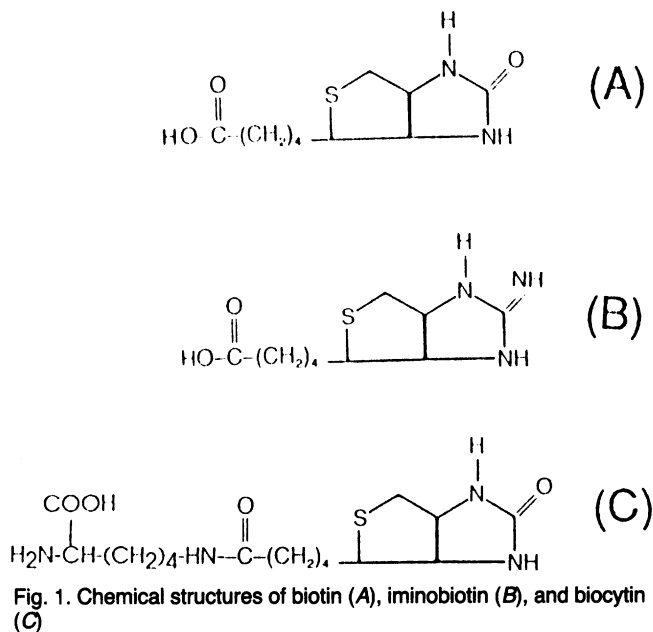


Fig. 1. Chemical structures of biotin (A), iminobiotin (B), and biocytin (C)

turers are using proprietary procedures to produce low-nonspecific-binding avidins suitable for specialized applications, e.g., avidin-D and avidin-DN (Vector Laboratories, Burlingame, CA).

The biotin-binding protein streptavidin also consists of four subunits but contains no carbohydrate; its pI is 5–6. Streptavidin is widely used in place of avidin because of its lower nonspecific binding. This protein is isolated from the culture broth of *S. avidinii* by ammonium sulfate precipitation, ion-exchange chromatography, and crystallization. Alternatively, iminobiotin columns can be used directly with the bacterial broth.

The streptavidin gene has recently been cloned and sequenced (22). From the complete nucleotide sequence, the amino acid sequence was deduced and compared with that of avidin. Many regions of the two molecules showed significant homology. The molecular mass of streptavidin was previously reported to be 60 kDa for the product isolated as described above by an initial ammonium sulfate precipitation (many commercially available products are prepared with such methods). From the gene sequence, a mass of 66 kDa was predicted; a 75-kDa product was isolated on iminobiotin columns. It is now known that native streptavidin of relatively high mass (66–75 kDa) can be converted to a lower-mass form (i.e., 60 kDa) by proteolytic digestion at both the N and C termini, which occurs during the ammonium sulfate precipitation step; this conversion is prevented if an iminobiotin column is used (18). The lower- M_r streptavidin is the commercially available product (called “core” or “truncated” streptavidin), which has better biotin-binding characteristics than the native protein. Native streptavidin can be converted to core streptavidin by a simple digestion with proteinase K (23).

Biotin

Biotin (Figure 1), also known as vitamin H, is present in minute amounts in every living cell. It acts as a co-factor of carboxylating enzymes, e.g., pyruvate carboxylase, which catalyzes the formation of oxaloacetate by condensation of pyruvate and CO_2 . The carboxyl group of biotin is the site of attachment of the molecule to ϵ -amino groups of lysine residues through an amide bond. During a carboxylation reaction, the CO_2 is first fixed to the iminogroup of biotin to form carboxy-biotin, which in a second step passes CO_2 to the substrate, e.g., pyruvate.

Numerous biotin derivatives are available and will be discussed in a separate section. Biocytin, an adduct of biotin and lysine (*N*- ϵ -biotinyl-L-lysine), is found naturally but also is synthesized from biotin and lysine. Biocytin derivatives prepared for labeling are mentioned below.

Biotinylation Procedures

One of the basic components in a biotin–avidin-based system is the biotinylated moiety, which can be a protein, a polysaccharide, a nucleic acid, a low- M_r substance, etc. To biotinylate such diverse classes of com-

Table 3. Major Biotin Labeling Strategies for Proteins and Carbohydrates

Molecule for biotinylation	Reactive group or moiety	Biotinylation reagent
Proteins, haptens, or peptides	Amine	NHS-biotin, NHS-LC-biotin
	Tyrosyl, histidyl	<i>p</i> -Diazobenzoylbiocytin
	Sulfhydryl	3-(<i>N</i> -Maleimidopropionyl) biocytin or iodoacetyl-LC-biotin
Glycoconjugates, carbohydrates (including antibodies and ribonucleotides)	Carboxyl	Carbodiimide and biotin hydrazide
	Vicinal hydroxyls	Periodate oxidation and biotin hydrazide

pounds, researchers have developed several different biotinylation reagents (active biotin derivatives). In addition, other biotinylation reagents have been developed for specific applications: e.g., cleavable reagents that can be used for biotinylation with the option of removing biotin at a later stage by reduction of a disulfide bond or hydrolysis of phenyl ester linkages (24–27).

An exhaustive review of many different biotinylation reagents has been published recently (28). Below, we describe biotinylation procedures for proteins, sugars, and nucleic acids. However, the same procedures can be used for other compounds bearing reactive groups. These procedures are summarized in Tables 3 and 4.

Proteins

In the vast majority of cases, proteins are biotinylated via the ϵ -amino groups of lysines by using an *N*-hydroxysuccinimide ester (NHS-ester) of a biotin analog (29).³ Figure 2 shows the two most commonly used reagents, along with a typical biotinylation reaction. The more recently developed reagent, NHS-LC-biotin, is the reagent of choice: it decreases steric hindrance because it has an extra spacer arm between biotin and protein. Consequently, the biotin is more exposed from the protein surface and is more available to bind avidin or streptavidin.

Biotinylation reactions with NHS-biotin derivatives (available commercially from many companies) are simple to perform. We use a reaction pH of 9.1 adjusted with 0.25–0.50 mol/L carbonate buffer. Proteins are used at a concentration of 0.1–2 g/L and, before biotinylation, are extensively dialyzed in 0.1 mol/L bicarbonate solution, to remove any interfering small molecules (e.g., amine-containing buffers). Biotin analogs, dissolved in a minimum volume of dimethyl sulfoxide, are added at 50- to

³Nonstandard abbreviations: NHS, *N*-hydroxysuccinimide; LC, long chain; dNTP, deoxynucleotide triphosphates; TdT, terminal deoxynucleotidyl transferase; PCR, polymerase chain reaction; AMPPD, 3-(2'-spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)-phenyl-1,2-dioxetane; NBT, 4-nitroblue tetrazolium; and BCIP, 5-bromo-4-chloro-3-indolyl phosphate.

Table 4. Biotinylation of Nucleic Acids

Method	Labeled nucleic acid	Principle
Nick translation	dsDNA	Enzymatic: DNase I and DNA polymerase I with biotinylated dNTP or their biotinylable precursors
Random priming	dsDNA	Enzymatic: Klenow enzyme, random sequence deoxyribonucleotides and biotinylated dNTP or their biotinylable precursors
Transcription	RNA	Enzymatic: RNA polymerase and biotinylated NTP with dsDNA template
Photobiotin Transamination	dsDNA, ssDNA, RNA ssDNA, dsDNA	Chemical bonding of photobiotin to nucleic acid Generation of biotinylable <i>N</i> ⁶ -substituted cytosines via sodium bisulfite and ethylenediamine
Psoralen derivatives	dsDNA with unlabeled ss regions	Intercalation of psoralen into dsDNA and interstrand link after irradiation
3'-end tailing	3'-end-labeled ssDNA or dsDNA	Enzymatic: TdT in the presence of biotinylated dNTP or biotinylable dNTP
Periodate oxidation	RNA	As in Fig. 3
T4 RNA ligase	RNA	Enzymatic 3'-end labeling with T4 RNA ligase and biotinylated ADP-derivatives
Linker or deoxythymidine phosphoramidite	Short 5'-end labeled ss synthetic oligonucleotide	Introduction of aliphatic amino group at 5'-end during automated phosphoramidite synthesis
Biotinylated nucleoside phosphoramidite	Short ss synthetic oligonucleotides labeled at any position	Introduction of biotinylated bases at any position during automated phosphoramidite synthesis
Nonnucleosidic biotinylated linker phosphoramidite	Short ss synthetic oligonucleotides biotinylated singly or multiply at their 5'-ends	Single or multiple introduction of nonnucleosidic biotinylated linker at 5'-end during automated phosphoramidite synthesis
PCR	dsDNA	Enzymatic incorporation of biotinylated nucleotides by Taq polymerase during PCR

ds, double-stranded; ss, single-stranded.

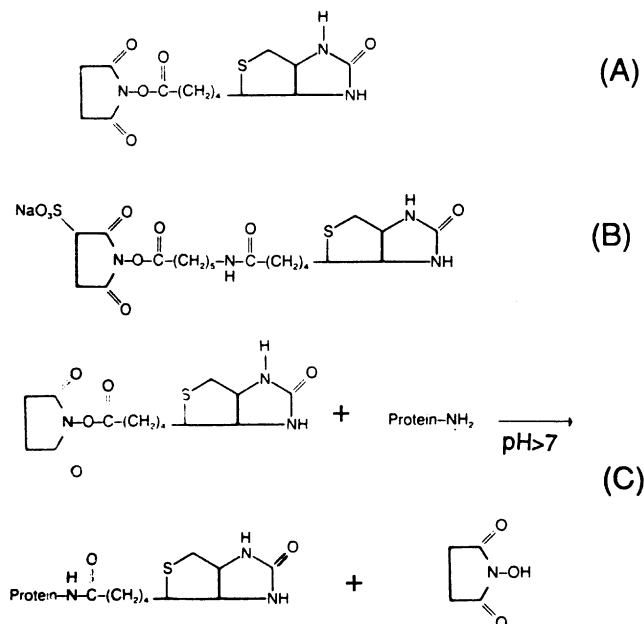


Fig. 2. Chemical structures of NHS-biotin (A) and NHS-LC-biotin (B) (C) A biotinylation reaction (protein + NHS-biotin). Multiple biotinylation occurs when the protein bears more than one $-NH_2$ group. The size of the molecules is not shown to scale

500-fold molar excess in relation to the protein concentration and incubated at room temperature for 1 h. The mixture is then used either as is or dialyzed against a 0.1 mol/L $NaHCO_3$ solution to remove unreacted biotin.

As is well documented, excessive biotinylation may inactivate a biologically active molecule. To avoid this, it is a good practice to perform trial biotinylations with

various amounts of excess biotin reagent to determine optimal performance. The extent of biotinylation does not usually need to be calculated; however, there are methods for doing so, and they are described in reference 30.

Alternative protein-biotinylation procedures are based on the targeting of groups other than the ϵ -amino groups of lysine residues. The reagent *p*-diazobenzoyl-biotin is specific for tyrosyl and histidyl amino acid side chains. Proteins that contain free $-SH$ groups can be biotinylated by using 3-(*N*-maleimidopropionyl) biocytin or iodoacetyl-LC-biotin. The same reagents can be used with proteins whose S-S groups are reduced to $-SH$ or proteins that are first thiolated. Carboxyl groups of proteins can also be used for biotinylation with biocytin hydrazide and a water-soluble carbodiimide reaction. Glycoproteins can be biotinylated through their sugar moieties by using biotin hydrazide or biotin-LC-hydrazide, as discussed further in the next section.

Carbohydrates

Carbohydrates, glycoproteins, or other glycoconjugates are easily biotinylated by using biotin-LC-hydrazide or biocytin hydrazide (31–33) (Figure 3). The glycoconjugate is first reacted with $NaIO_4$ to oxidize vicinal hydroxyl groups of the sugars to aldehyde groups. These groups are then allowed to react with biotin hydrazide, biotin-LC-hydrazide, or biocytin hydrazide to produce the biotinylated sugar moiety. This procedure has been used only occasionally to biotinylate antibodies. One group (26) found that this procedure gave inferior results in comparison with biotinylating

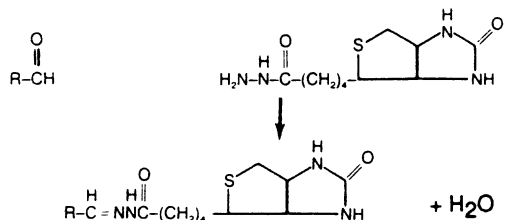


Fig. 3. Reaction of an aldehyde, generated from the oxidation of a *cis*-diol by NaIO_4 , with biotin hydrazide

antibodies through the ϵ -amino groups. In our hands, biotinylation of antibodies with NHS-LC-biotin yielded a twofold greater signal in time-resolved fluorometric immunoassays involving labeled streptavidin than did the same antibodies labeled via sugar moieties with biotin-LC-hydrazide. In addition, in biotinylation of other glycoproteins, e.g., bovine thyroglobulin, we have found that, during the NaIO_4 oxidation step, the aldehyde groups generated react with amino groups of the protein, resulting in protein polymerization.

Nucleic Acids

Nucleic acid biotinylation can be accomplished with several different procedures, most of which have evolved very recently. In general, cloned nucleotide fragments are biotinylated with different strategies from those used with small synthetic oligonucleotides. The major interest in biotinylating nucleotides arises mainly from the need to devise highly sensitive nonisotopic hybridization assays.

Many procedures for nucleic acid biotinylation are based on enzymatic catalysis; others are chemical methods. Enzymatic biotinylation procedures rely on the availability of biotinylated nucleotide analogs that can act as enzyme substrates. Alternatively, nonbiotinylated nucleotide derivatives can be used as substrates but, after they are incorporated into DNA, they can be chemically biotinylated because they carry into their structure an easily biotinylable group (e.g., an aliphatic primary amine group).

The first reported biotinylated nucleotides were biotinylated uridine triphosphate (UTP) and deoxyuridine triphosphate (dUTP) (Figure 4) (34, 35). Biotin is attached to the 5-position of uridine base through an allylamine linker. The distance of biotin from the nucleotide base, measured in numbers of atoms, is shown in the name of the molecule, as biotin-*n*-base, e.g., biotin-11-dUTP and biotin-16-dUTP. More recently, biotinylated dATP and dCTP with linkers of between three and 17 atoms long have been described (36). Precursors of these molecules are modified bases that contain linker arms with free $-\text{NH}_2$ groups, e.g., N^6 -(6-aminohexyl) dATP (Figure 4), and are thus easily biotinylated with NHS-LC-biotin. Many biotinylated nucleotides and some of their precursors are commercially available. Another useful class of biotinylated nucleotides consists of nucleotides that contain the S-S group within the structure of the linker arm. These "releasable" nucleotide analogs can be used in applications where the

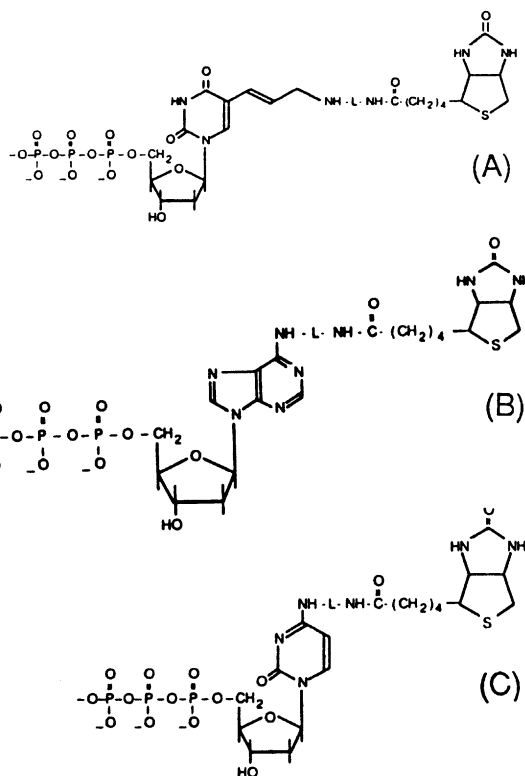


Fig. 4. Biotinylated deoxynucleotide triphosphates

(A) dUTP derivatives. *L* can be a variable structure; e.g., $L = \text{CO}(\text{CH}_2)_5$ will yield biotin-11-dUTP, and $L = \text{CO}(\text{CH}_2)_2\text{SS}(\text{CH}_2)_2$ will yield biotin-12-SS-dUTP. (B) dATP derivatives. $L = (\text{CH}_2)_6$ yields biotin-7-dATP. When $L = (\text{CH}_2)_6\text{NH}_2$, a biotinylable precursor of dATP is generated. (C) dCTP derivatives. $L = (\text{CH}_2)_6$ yields biotin-7-dCTP. Adapted by permission from ref. 12

nucleic acid needs to be released after its binding to streptavidin (25, 37).

The biotinylated nucleotide analogs or their precursors, e.g., N^6 -(6-aminohexyl) dATP, can be introduced into DNA by using two general and well-established enzymatic procedures: nick translation and random priming. In nick translation, relatively large (>1 kb) double-stranded DNA is allowed to be attacked by the enzyme DNase I, which creates "nicks" (holes) at random points of one DNA strand. A second enzyme in the mixture, *E. coli* DNA polymerase I, repairs the nicks by both its 5' → 3' exonuclease activity (to chew off more nucleotides) and its 5' → 3' polymerase activity (to fill in the gap). During the 5' → 3' polymerization, the biotinylated nucleotides or precursors in the reaction mixture are incorporated into the DNA. Although in theory one can incorporate more biotins by using only biotinylated dNTP in the reaction mixture, this is not usually attempted for two reasons: the highly biotinylated DNA may not be suitable to use as a probe (because it may not reassociate with the target with acceptable specificity, affinity, and kinetics), and incorporation of >40 biotinylated nucleotides per kilobase of DNA does not improve the sensitivity of the assays developed (probably because of steric hindrance problems associated with streptavidin binding) (36). We have also seen this effect with highly biotinylated proteins, e.g., antibodies labeled with >20 biotins. Nick translation not only labels

but also fragments the original DNA. The ratio of the enzymes DNase I and DNA polymerase I and the incubation time and temperature of the reaction are adjusted so that the labeled probes are 500–1500 bases long. Nick translation kits are commercially available from several manufacturers, making the procedure easy and reliable to apply even by nonspecialists. In these kits, usually one biotinylated nucleotide (e.g., biotin-7-dATP) is incorporated into the reaction mixture along with three unlabeled nucleotides.

The random priming method was originally developed to label DNA radioactively (38); it can be used with probes <500 bp long. The method is based on the denaturation of DNA by heating and the subsequent annealing to the DNA, upon cooling, of random-sequence oligodeoxyribonucleotides. The DNA-random oligonucleotide hybrids are substrates for the Klenow fragment of *E. coli* DNA polymerase I, which extends in the 5' → 3' direction, incorporating nucleotides and any biotinylated nucleotides in the newly synthesized strands. Again, random priming kits are commercially available and are extremely easy to use. Protocols involving biotinylated nucleotides (e.g., biotin-11-dUTP, which is incorporated as if it were dTTP, or biotin-7-dATP) are also available from these manufacturers.

In any case involving a precursor of biotinylated nucleotides, e.g., *N*⁶-(6-aminohexyl) dATP, the product can be easily biotinylated through use of NHS-LC-biotin.

Cloned double-stranded probes inserted into transcription vectors that contain the promoters for SP6 and T7 RNA polymerase can be transcribed in the presence of biotinylated ribonucleotide triphosphates to produce single-stranded biotinylated riboprobes.

Labeling of DNA can also be achieved by other procedures. A new biotinylation reagent, photobiotin (Figure 5) (39), is now commercially available and has been used to label not only DNA but also proteins and RNA (40). Photobiotin contains the biotin moiety attached to a photoactive aryl azide group through a linker. Upon exposure to intense visible light (350–370 nm) for 15–20 min, the aryl azide group is converted to a highly reactive aryl nitrene, which reacts and biotinylates the DNA. The biotin incorporation is about 5–10 biotins per kilobase of DNA. The bond is very stable, although its exact nature is not known.

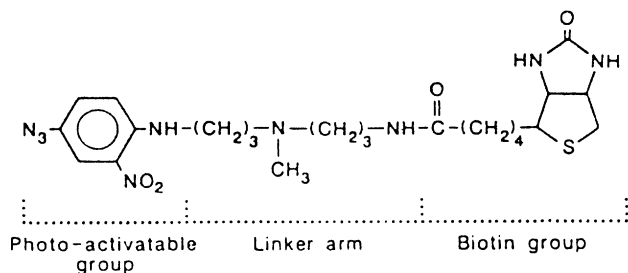


Fig. 5. Chemical structure of photobiotin
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Another method used to label DNA chemically is based on the well-known transamination reaction of cytosine residues with sodium bisulfite and a diaminoalkane, e.g., ethylenediamine (Figure 6) (41). This reaction works with single-stranded DNA (prepared by heating double-stranded DNA at 100 °C for 3 min) in one step in the presence of both bisulfite and ethylenediamine. The end product (*N*⁴-substituted cytosine) can then be easily biotinylated by using an NHS-ester of biotin (see Figure 2). The biotin incorporation with this method can be as great as 30–100 biotins per kilobase of DNA and can be controlled somewhat by adjusting the pH and the bisulfite concentration in the reaction. In a variation of this technique, DNA can be labeled by reacting the cytidine residues with biotin hydrazide in the presence of sodium bisulfite (42).

Psoralen derivatives of biotin (Figure 7) have also been used as labeling reagents for DNA (43–45). Psoralen has the ability to intercalate into double-stranded nucleic acids and, when irradiated with light (360 nm), form interstrand crosslinks. Probes suitable for labeling with psoralen–biotin derivatives must have an appropriate structure: a single-stranded region that will hybridize to the target and a double-stranded region that will carry the label. Usually, the psoralen moiety and biotin are separated by a spacer arm (Figure 7) to increase the availability of biotin for streptavidin binding. About one biotinylated psoralen is present in every 10 bp in the double-stranded region of the probe.

In recent years, it has been possible to synthesize short (<50 bases) single-stranded oligonucleotide probes by automation and to use these probes for hybridization or as primers for the polymerase chain reaction (PCR) or sequencing. These probes can be biotinylated via several different procedures. For end-labeling at the 3'-end, one can use the enzyme terminal deoxynucleotidyl transferase (TdT) and biotinylated nucleotides, e.g., biotin-11-dUTP (46). Alternatively, TdT can be used to introduce 4-thiouridine, which can then be reacted with haloacetamido derivatives of biotin, e.g., iodoacetyl-LC-biotin, to biotinylate the probes (47). RNA probes can also be labeled with biotin at their 3'-termini by first reacting the 2',3'-*cis*-hydroxyls of the terminal ribose with periodate, to produce aldehyde groups that can then be reacted with biotin hydrazide as described above (Figure 3) (48). RNA probes have also been biotinylated at their 3'-termini by use of the enzyme T4 RNA ligase (49). End-labeling at the 5' end

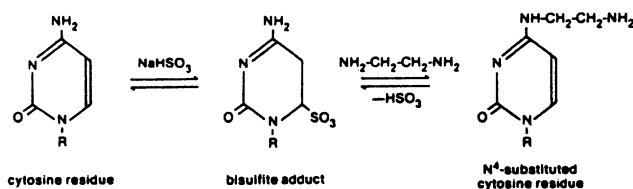


Fig. 6. Bisulfite-catalyzed transamination reaction with ethylenediamine
The *N*⁴-substituted cytosine residue is biotinylated with NHS-LC-biotin as shown in Fig. 2

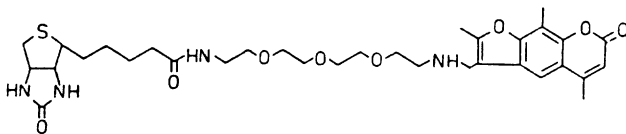


Fig. 7. Biotinylated psoralen used to label DNA
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of DNA has also been achieved with periodate oxidation (50): ribonucleotide monomers can be incorporated into short synthetic oligonucleotides by either the phosphotriester or the phosphoramidite routes and biotin-labeled with periodate oxidation and biotin hydrazide. Agrawal et al. (50) also synthesized another monomer (linker phosphoramidite), which could be incorporated at the 5'-end of a synthesized oligonucleotide. After deprotection, an $-NH_2$ group at the 5'-end of the oligonucleotide could be easily biotinylated with NHS-LC-biotin.

Many other methods can incorporate a free biotinylable amino group at the 5'-end of an oligonucleotide. For example, Smith et al. (51) used automated phosphoramidite chemistry to introduce deoxythymidine containing a linker arm with aliphatic amine.

By using the TdT tailing reaction, one can attach multiple biotins to the 3'-end of a synthetic polynucleotide. The products have greater sensitivity than probes singly biotinylated at their 5'-ends (46, 52).

Newer biotinylation methods allow for biotin introduction internally into an oligonucleotide. For example, Jablonski et al. (53) and Haralambidis et al. (54) reported the synthesis of C-5-substituted deoxyuridines carrying a masked primary aliphatic amino group. These modified bases can be used as phosphoramidites in automated oligonucleotide synthesis (52); after deprotection, they possess a free amino group available for biotinylation. For better availability, this amino group should be attached to the base through a linker arm. With such procedures, both the location and the number of amino groups can be controlled. Similarly, C-8-substituted deoxyadenosine phosphoramidites have been reported (55). In yet more-straightforward procedures, biotinylated nucleoside phosphoramidites were synthesized (56, 57) and used to produce by automated synthesis oligonucleotides that were multiply labeled at any site. More recently, nonnucleosidic phosphoramidite linker units carrying biotin have been synthesized and used to produce multiply biotinylated (e.g., eight biotins) short probes (58).

Cook et al. (52) examined in detail the achievable sensitivity in hybridization assays involving short 17-mer probes labeled with either one or up to three biotins at various sites internally or externally to the probe. They concluded that probes tailed with TdT gave the best overall results, whereas the poorest results were obtained with internally labeled probes. They also found that probes with internal biotins form less-stable hybrids than probes with external biotins. Others (58) also noticed that multiple biotinylation of short probes does not improve signal yield when streptavidin is the carrier of the label.

PCR is also a convenient method of producing relatively short labeled probes (59). During the PCR process, labeled nucleotides can be introduced into the final probe product. The enzyme Taq polymerase used in the PCR process incorporates biotinylated nucleotides, among others.

The degree of biotinylation of DNA, produced by any of the methods described above, can be assessed by at least two recently described procedures (60, 61).

The Avidin-Biotin Interaction as a Detection System

The avidin-biotin interaction can be used for detecting a diverse number of targets with three different basic configurations (62-64):

(a) Avidin or streptavidin is labeled with a detectable molecule, e.g., an enzyme, fluorescent, chemiluminescent, or radioactive probe; a metal; or some other moiety. Biotin present in another reactant (antibody, nucleotide, Protein A, lectin, etc.) links the target molecule with the labeling system (Figure 8A). This detection format is used widely for immunoassays, DNA hybridization assays, immunohistochemistry, and flow-cytometry. Avidin and streptavidin conjugates carrying a variety of detectable molecules are commercially available (Table 2).

(b) Streptavidin is used unlabeled and serves to link the biotinylated binder with the biotinylated detection molecule (Figure 8B). This variation of the system takes advantage of the multiple biotin-binding sites in each avidin or streptavidin molecule. It is also used widely in immunoassay and DNA hybridization techniques, especially with probes that can be easily biotinylated (e.g., enzymes and proteinaceous fluorescent molecules).

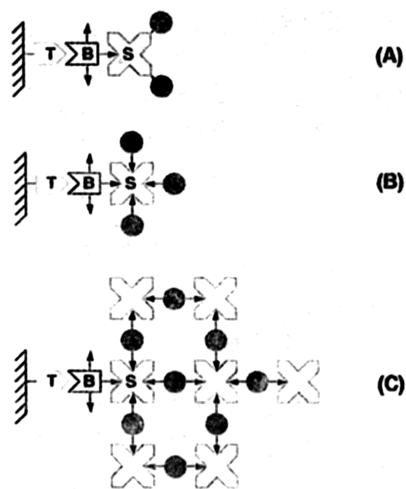


Fig. 8. Basic configurations of the biotin-streptavidin system

In all cases, the target molecule (T) is shown immobilized on a solid support. (A) The specific binder (B) of the target is biotinylated (\rightarrow), and streptavidin (S) carries a label (O). (B) Same as (A) but streptavidin is used unlabeled to link the biotinylated binder and the biotinylated label. (C) Unlabeled streptavidin is mixed with biotinylated label to form a polymeric complex with some residual biotin-binding activity. This polymer binds to the biotinylated binder as in (A)

(c) The third method combines effectively the principles of (a) and (b) to yield a significantly more sensitive system (3). The basic idea is to mix, under controlled conditions, unlabeled streptavidin or avidin and a biotinylated detection reagent, e.g., an enzyme. Given the multiple biotin binding sites on avidin or streptavidin, one can generate a polymer having some free biotin-binding sites. This reagent is then used as in (a) but affords superior sensitivity. Kits offering the streptavidin or avidin and biotinylated enzymes at optimized concentrations, so that one can form the complex by simply mixing the reagents, are commercially available and are known as ABC (avidin-biotin complex) kits.

The Avidin-Biotin Interaction as an Affinity System

Immobilized avidin or streptavidin can be used to retrieve, purify, and characterize various moieties of interest (2). For example, a protein present in cells can be reacted with a specific biotinylated probe (antibody, receptor, lectin, hormone, etc.), which can then be retrieved by being placed in contact with a column or magnetic beads with immobilized avidin or streptavidin. The protein of interest can be released by disrupting the protein-biotinylated probe complex (2, 26, 65, 66). This approach can also be used with cleavable biotin analogs, facilitating easy release of the bound complex (25, 37). Biotinylated DNA can be retrieved by using similar procedures.

Avidin- or streptavidin-coated solid phases have been used effectively as separation agents in heterogeneous immunoassays (67, 68) and in DNA-hybridization assays (69). Two examples are given in Figure 9. Other applications include isolating and purifying DNA-binding proteins by use of streptavidin-DNA-biotin solid-phase (70), solid-phase DNA sequencing in combination with PCR (71), separation of cells tagged with biotinylated antibodies (72), and efficient fusion in hybridoma production (73) (see below).

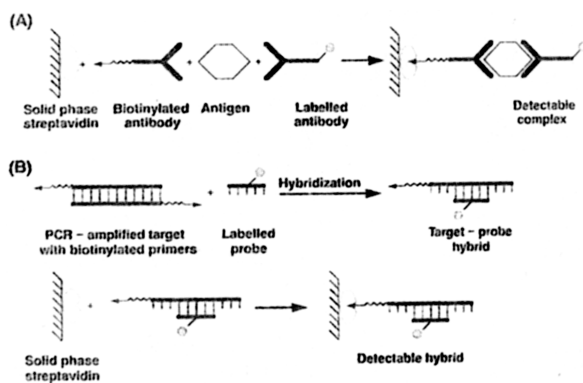


Fig. 9. Immobilized streptavidin used as a separation agent in immunoassays (A) and nucleic acid hybridization assays (B). Washing removes unreacted detection antibody or labeled probe (not shown).

Selected Applications of the Biotin-(Strept)Avidin System

Immunological Assays

With the existence of biotinylation reagents and various avidin- or streptavidin-based detection systems (Tables 1 and 2), one would expect that many manual or automated avidin-biotin based immunoassays would be available commercially. This is not the case, however, and most of the available immunoassay kits or methods rely on direct labeling of the detection antibodies of interest. This is generally advantageous through avoiding the extra step of adding streptavidin label. On the other hand, the versatility of the avidin-biotin system makes it very attractive for research applications, as has been recently stressed in the literature (74), for the following reasons: For research applications, when appropriate antibodies are available, one of them can be immobilized on a solid support (e.g., polystyrene microtiter wells) and the other can be easily biotinylated as described before; streptavidin-enzyme conjugates, as well as many enzyme substrates, or other conjugates are available from many manufacturers; with such assay configurations (Figure 8A), excellent sensitivity can be achieved because of the amplification introduced by the avidin-biotin system. This amplification can be as high as 100-fold (10-20 active biotins per antibody and three to six enzymes per streptavidin). Competitive-type immunoassays can also be devised by using either the immobilized antigen approach and biotinylated antibody (75, 76) or the immobilized antibody approach and biotinylated antigens (77). In our hands, if good antibodies and calibration material are available, excellent assays can be set up in a few working days.

The most widely used enzyme conjugates for immunoassay are streptavidin-horseradish peroxidase (EC 1.11.1.7) and streptavidin-alkaline phosphatase (EC 3.1.3.1). With peroxidase, many substrates that yield colored products can be used (78). With alkaline phosphatase, the colorimetric substrate *p*-nitrophenyl phosphate, the fluorogenic substrate 4-methylumbelliferyl phosphate (79), or the chemiluminogenic substrate 3-(2'-spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy) phenyl-1,2-dioxetane, disodium salt (AMPPD) (80, 81), is used, the latter two having the better sensitivity. Recently, streptavidin conjugates have been used in time-resolved fluorometric applications (82-89). In one variation, Eu^{3+} is noncovalently attached to streptavidin by a strong Eu^{3+} chelator of the aminopolycarboxylic acid type (82, 83). After the immunological reaction is completed and excess biotinylated antibody is washed off, Eu^{3+} -labeled streptavidin is added (Figure 8A). The Eu^{3+} on the complex is then released in solution by lowering the pH and is re-complexed with ligands to form fluorescent complexes of long fluorescence lifetime. These complexes are quantified by using time-resolved fluorometry.

A different approach involves labeling streptavidin with the europium chelate of 4,7-bis(chlorosulfonyl)-1,10-phenanthroline-2,9-dicarboxylic acid (84-89).

Streptavidin carrying a variable load of these chelates, ranging from 14 to 480 per molecule, has been synthesized and used to devise highly sensitive time-resolved fluorescence immunoassays.

Recently, Ishikawa et al. (90) used the avidin-biotin system to devise noncompetitive immunoassays for haptens. They biotinylated the hapten, which could then be bound by both its specific antibody and labeled streptavidin. Daunert et al. (91) devised a homogeneous assay for biotin, based on the observation that biotinylated glucose-6-phosphate dehydrogenase is inhibited if bound to avidin.

A very interesting variation of the avidin-biotin system in immunoassay has been developed by Bobrow et al. (92). In this assay, called catalyzed reporter deposition, horseradish peroxidase is used as label in a typical "two-site" immunoassay. However, the peroxidase, instead of producing a measurable signal, is used to catalyze the deposition of biotin-derivatives on the solid-phase, which is then detected by using streptavidin-enzyme conjugates. This approach amplifies manifold the signal generated and shows promise for future applications that require very high sensitivity.

Many commercial sources apply the biotin-avidin technology not as a detection technique but as a high-affinity separation method of bound and free labeled reagent (Figure 9) (67, 68).

Nucleic Acid Hybridization Assays

The dominant labeling systems for various DNA-based assays are still radionuclides, i.e., ^{32}P and ^{35}S . These radionuclides offer superior sensitivity to that of the nonisotopic alternatives available today (93). However, ^{32}P and other radionuclides have some major disadvantages. Many laboratories, especially those interested in routine applications in the field of infectious disease, genetic disease, forensics, and cancer, are exploring alternative systems. Nonisotopic methodologies are now entering nucleic acid-based testing, with excellent prospects. In many applications (e.g., infectious disease), extreme sensitivity is not always needed; moreover, with the advent of PCR, the amplified product can be easily detected with nonisotopic methods. Some new nonisotopic assays claim to be as sensitive as ^{32}P assays (94, 95).

Because biotin can be easily and multiply incorporated into DNA without altering its ability to hybridize with its targets, it is the label of choice in many nonisotopic systems, with conjugated streptavidin being used for detection. Streptavidin-alkaline phosphatase conjugates have been widely used in combination with the colorimetric reagent NBT-BCIP (94), but more recently the chemiluminometric reagent AMPPD has been used for more-sensitive assays (80, 95). Streptavidin labeled with europium in combination with time-resolved fluorometry has also been used (83), or more recently, streptavidin labeled with europium chelates (95). From the information given before, one can easily deduce that any type of DNA assay (Southern, Northern, Western, dot-blot, etc.) can be performed by using

the biotin-avidin interaction: biotinylated probes for Southern and Northern blotting and biotinylated antibodies for Western blotting.

Other Applications

The application of the system for DNA sequencing has recently been reported in different formats (71, 96). In one assay (96), based on the dideoxy chain termination sequencing method, biotinylated primers are used with direct blotting electrophoresis, which facilitates the transfer of the generated DNA fragments from the polyacrylamide gel to a nylon membrane (97, 98). The fragments, all containing the biotinylated primer, are then made visible by using streptavidin-alkaline phosphatase and the NBT-BCIP substrate.

In flow cytometry, biotinylated antibodies are used to react with specific membrane targets; the cells are made fluorescent by further reaction with streptavidin-fluorochrome conjugates. In receptor-hormone studies, the hormone can be biotinylated, provided biotinylation does not alter its ability to bind to the receptor (99). In electron microscopy, ultrastructure can be localized by using specific antibodies and avidin-gold or avidin-ferritin conjugates or unlabeled streptavidin and biotinylated ferritin (31, 100).

More recently, some new reagents have been described for use with the avidin-biotin system. Antibiotin antibodies can be used when the high affinity of the avidin-biotin interaction is not desirable (35). In this case, the biotin moiety is used as a hapten in a manner similar to other "hapten-anti-hapten" systems, e.g., the digoxigenin system (101). Antibodies against avidin and streptavidin have also been prepared and are available conjugated with enzymes or biotinylated. Biotinylated anti-avidin and anti-streptavidin are interesting reagents because they can react with avidin or streptavidin from two different sites. They can thus be used as amplifiers, as described elsewhere (102-105).

Another novel application of the biotin-avidin system has been the facilitated cell fusion for hybridoma production (73). In this method, biotin is attached to the membrane of myeloma cell lines used for hybridoma production. An antigen-avidin conjugate is mixed with the spleen cells suspension that is producing the antigen-specific antibodies. The spleen cells producing antibodies express the antibodies on their surfaces and are selectively labeled with avidin. Mixing the biotinylated myeloma cells and the avidin-labeled antibody-producing spleen cells yields complexes of the form myeloma cell-biotin-avidin-antigen-spleen cell, which will fuse efficiently by electrofusion. The close proximity of myeloma cells with antibody-producing spleen cells (but not non-antibody-producing spleen cells) facilitates greater yields during electrofusion. Alternatively, the myeloma cells can be avidinylated and the antigen of interest can be biotinylated and attached to the antibody-producing spleen cells.

Other interesting applications of the system for targeted imaging and drug delivery in combination with specific antibodies have been reported (10, 106).

In conclusion, the extreme versatility of the avidin-biotin system and the commercial availability of many auxiliary reagents will undoubtedly contribute to the more widespread use of this system in diverse areas of biotechnology.

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