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Photoaffinity Labeling for Structural Probing Within Protein

Foreword

Harnessing the Protein

The highly ordered molecular assembly of living systems represents an endless frontier for the application of chemical biology. A useful chemical approach to define specific molecular recognition is covalent cross-linking of a ligand and its receptor in an affinity-based manner. The chemical basis for affinity cross-linking is classified into two categories: ground-state reactions secure the residue-selective nature of affinity labeling and excited-state reactions generate highly reactive species to characterize the powerful feature of photoaffinity labeling. The major advantage for photoaffinity labeling is that the probe is inert before irradiation to prevent ground-state side reactions with surrounding molecules in a non-specific manner. Ideally, the irradiation immediately generates an extremely reactive species to complete specific and stable cross-links which comes in contact with any one of the residues located close by. Since Frank H. Westheimer originally introduced this unique idea of the method in 1962, photoaffinity labeling remains and should continue to be a principal chemical method for the identification of a particular target among the complex mixture of biomolecules.

Photoaffinity labeling has become increasingly important in association with the development of rationally designed powerful probes. Simply stated, probes are prepared by installing a photoactivatable functional group (photophore) on the framework of biological ligands. For target-specific labeling, probes are always needed to satisfy conflicting requirements. The probe structure should be close to the original ligand whereas the introduction of the photophore and detection tag cause an increase in the size of probes resulting in the decrease of affinity. For designing probes, the generated reactive species should react rapidly within the functional domain depending on the affinity between the ligand and the target but should not indiscriminately react with co-existing molecules.

This book is composed of 12 chapters that describe recent topics of photoaffinity labeling mainly by taking advantage of the use of diazirine photophore. Leading experts have written their chapters by focusing on the photophore's application for illuminating the interesting protein world. The first three chapters describe the

rational design of efficient probes, followed by two chapters that consider the rapid and specific approach for the analysis of trace photo-labeled products. Chapters introducing synthetic, chemoselective, and genetic approaches for installing the diazirine photophore suggest new aspects of the methodology for the elucidation of bio-molecular assembly. Successful applications for glucose transporter and peroxisome represent the power of the methodology to analyze their structure and function. The last two chapters introduce the recent topic of photoaffinity labeling in the process of drug discovery and development.

Since the first report of photoaffinity labeling, the probe has been continuously improved through several important innovations to unleash the full power of the methodology. Now, photoaffinity labeling is well defined to ensure its specificity on labeled sites, which reveals the method to be an important approach of chemical biology for entering important areas of life science. An interesting application should be the structural probing of proteins that are difficult to crystallize. Photoaffinity labeling also could be a reliable strategy to reveal the molecular target during the early stage of drug discovery and development.

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Contents

Chapter 1
Multifunctional Photoprobes for Identification of Ligand Sites Within Biomolecules

Makoto Hashimoto

Abstract The technique of photoaffinity labeling has become increasingly appreciated as a powerful methodology for post-genome field because it is one of the attractive methods to elucidate the interactions between bioactive ligand and biomolecule. The combinations with detection and isolation methods are essential to identify photolabeled components. There are several methods to introduce detection and isolation methods, which are so-called "tag," for photolabeled components from the photoaffinity label mixture. High detection limits of tag enable us to identify the photolabeled components. The introduction of detection and isolation tags in the ligand skeleton is one of the ways to archive identification of photolabeled components because the labeled components have been only introduced the tag. On the other hands, the specific biological interactions for target biomolecules are also utilized to identify photolabeled components. The chapter summarized that the several combinations of photoaffinity labeling and "tag" to study labeled components effectively.

Keywords Photoaffinity labeling • Chemical biology • Ligand binding sites • Radioisotope • Immunochemistry • Avidin–biotin

1.1 Introduction

Elucidation of protein functions based on the structure–activity relationship is one of the greatest interests for scientists to reveal the mechanisms of homeostasis functions in life. In the human body, many proteins are activated and/or inactivated by various ligands to maintain homeostasis. Understanding the mechanism

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of molecular interactions between small bioactive ligands and proteins is an important step in rational drug design and discovery. For these purposes, a genetic approach provides an efficient and indirect route for pinpointing functional amino acids within proteins by deleting or mutating the native amino acid alignment of proteins. When the target protein can be expressed in a large quantity, 3D structural determinations which include NMR spectroscopy and homology modeling are a powerful and direct approach for analyzing the three-dimensional structure of proteins at the atomic level. Chemical methods, which are fundamental in chemical biology, provide an alternative route for the direct identification of target proteins in biomolecule mixtures as well as their ligand binding site structure because these analyses are based on the affinity between the ligand and target protein. Affinity-based chemical modification introduces a useful tag for analyzing the target protein. For example, single molecular imaging with fluorophore (Wallrabe and Periasamy 2005) visualizes target biomolecules in complex systems for imaging the localization of biomolecules and processing the flow of bioactive compounds in cell compartments. For specifically attaching the desired tag on the target protein, affinity labeling (Wold 1977) has a limited role because the method requires the presence of nucleophilic residues near to a ligand binding site for preventing nonspecific introduction of the tag to a site different from the binding site or on other coexisting biomolecules. But recent progress of molecular biology prompted site-directed labeling using cysteine mutants to overcome this problem (Foucaud et al. 2001).

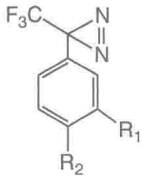
Photoaffinity labeling (PAL) (Brunner 1993; Kotzyba-Hibert et al. 1995; Hatanaka et al. 1996a, b; Dormán and Prestwich 2000; Hashimoto and Hatanaka 2008a) largely increased the capability of specific tagging. Photochemically generated highly reactive species (Chap. 2) introduce covalent bonds between ligand and protein in a nonselective manner, and any amino acid in the binding site can be tagged.

One of the successful applications of photoaffinity labeling is the identification of target biomolecules in crude extracts using radioisotope-labeled probes as highly sensitive detection tags. The covalent bond fixes the tag to the contact point even though affinity was destroyed by the denaturation condition, which allows further sophistication in detailed structural analysis, and the detection of tagged components may reflect the status of conformational changes of target molecules.

The other future is to identify the labeled regions of target biomolecules, and combinations with specific purification methods for labeled components are needed (Hatanaka et al. 1996a, b; Hashimoto and Hatanaka 2008a, b; Tomohiro and Hatanaka 2014; Hatanaka 2015).

Here we briefly describe the fundamentals of several concepts.

1. PAL with radioisotope tag
2. PAL with immunological methods
3. PAL with avidin–biotin system



1 R_1 = H, R_2 = CT_2OH
2 R_1 = T, R_2 = COOH
3 R_1 = OCHTCOOH, R_2 = H
4 R_1 = OCT_3, R_2 = $COOCH_3$
5 R_1 = H, R_2 = ^{14}COOH
6 R_1 = NO_2, R_2 = $OCH_2{}^{14}COOH$
7 R_1 = $O^{14}CH_3$, R_2 = COOSu
8 R_1 = ^{125}I, R_2 = H
9 R_1 = ^{125}I, R_2 = CH_2OH
10 R_1 = NCS, R_2 = ^{125}I

Fig. 1.1 Radioisotope contained phenyldiazirines. **1** (Latli et al. 1998), **2** (Ambroise et al. 2001), **3** (Rimoldi et al. 1993), **4** (Hatanaka et al. 1994a, b), **5** (Bender et al. 2007), **6** (Hatanaka et al. 1992, 1994a, b), **7** (Hatanaka et al. 1995), **8** (Brunner and Semenza 1981), **9** (Blanton et al. 1998, 2000), **10** (Dolder et al. 1990)

1.2 PAL with Radioisotope Tag

Radioisotope is one of the highest detection methods for low amounts photolabeled component (Filer 2009). Tritium (Latli et al. 1998; Ambroise et al. 2001; Rimoldi et al. 1993; Hatanaka et al. 1994a, b), ^{14}C (Bender et al. 2007; Hatanaka et al. 1992, 1994a, b, 1995), and ^{125}I (Brunner and Semenza 1981; Blanton et al. 1998, 2000; Dolder et al. 1990) were well selected for these purposes. Several radioisotope-labeled phenyldiazirine derivatives are summarized in Fig. 1.1. The introduction of radioisotope elements must be considered in the late synthetic stage. Researchers have to perform pre-experiment with unlabeled reagents to well-handled hole of experiments. Major withdraws to utilize radioisotope are limited amount of radiolabeled reagents for derivatizations. It was well observed that chemical synthetic yields in radioisotope are less than twice than in unlabeled synthetic methods because less amount reagents are available in radioisotope methods. For example, methylation of diazirinyl phenolic alcohol with methyl iodide was very effective in unlabeled reaction but was less than 30% with radiolabeled methyl iodide to afford 4 (Hatanaka et al. 1994a) or 7 (Hatanaka et al. 1995). Furthermore, purification steps (Preparative TLC and HPLC) for less amount compounds sometimes drawback to utilize radioisotope methods. Radioisotope was very useful as detection tag, but was less effective for isolation tag for photolabeled components due to overlap with unlabeled components in the purification steps. The details on applications are mentioned in a later chapter (Chaps. 4 and 5).

The recent progress of mass spectrometry for biomolecules promoted researchers to apply stable isotope for photoaffinity labeling. Detailed synthesis (Chap. 6) and applications (Chap. 2) are summarized in the later chapters.

1.3 PAL with Immunological Systems

Immunological methods are very useful to purify the photolabeled components with antibody, which consisted with target molecules. Immunoprecipitates were applied for photolabeled components and the antibody recognized the peptides derived

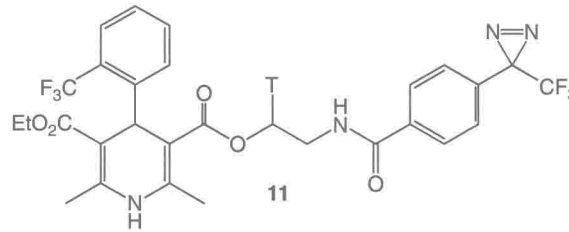


Fig. 1.2 [^{3}H]-Diazepam **11** for photoaffinity labeling of calcium channel

from both unlabeled and photolabeled biomolecules. Furthermore radioisotope introduced photolabeled ligand to detect low limit amounts.

These strategies were applied to elucidation of binding site of the ligands to ion channels. Antibody was prepared for predicted peptide sequences, and utilized photolabeled mixture for target ion channel with radiolabel. The correct predictions were caused immunoprecipitates, which contained radioisotopes. These experiments revealed binding site of [^{3}H]-diazepam **11** to calcium channels (Fig. 1.2, Nakayama et al. 1991, 1993).

It is very effective and the predictions of ligand binding site to biomolecules were easy to estimate because of its primary sequence. The details on applications are mentioned in later chapters (Chap. 5).

But sometimes it is difficult to apply these methods for unknown sequences for target biomolecules.

An antibody for the ligand skeleton is also available for the detection of the labeled components. Anti-galactosylceramide and anti-ceramide antibody was utilized to detect diazirinyl containing ceramide derivatives on TLC or PVDF membrane (Hashimoto and Hatanaka 2004).

1.4 PAL with Avidin–Biotin System

It is essential for predicted sequence to detect photolabeled components with immunological methods. It is very difficult to apply this method to unknown biomolecules. Specific interaction between avidin and biotin has been well studied. Avidin, which consists of tetrameric subunits, recognizes small molecule biotin with very strong affinity ($K_d = 10^{-15}$ M) (Bayer and Wilchek 1990). Other biotin-specific recognized biomolecules are streptavidin, anti-biotin antibody, and monomeric avidin. Streptavidin (Bayer et al. 1990) is purified from *Streptomyces avidinii* and has the same affinity for biotin as avidin, but the pI of the protein is between 5 and 6, whereas the pI of avidin is almost basic (pI 10).

Furthermore, streptavidin is non-glycosylated. These differences suppress the unspecific binding of biotinylated compounds to streptavidin rather than avidin. The strong interaction between (strept)avidin and biotin was very useful for detection of less amounts (10^{-13} mol) of biotinylated biomolecules with high detection methods (i.e., chemiluminescence, fluorescence, etc.) to the same degree as radioisotope detection. Researchers also utilized anti-biotin antibody

(Dakshinamurti and Rector 1990), which is less sensitive than (strept)avidin ($K_d = 10^{-9}$ M), to detect biotinylated components when it is too hard to make (strept)avidin–biotin complex.

Monomeric avidin, which is only prepared on a solid support, has less affinity than avidin ($K_d = 10^{-9}$ M) (Green and Toms 1973), and dissociation of the avidin–biotin complex is easier than the tetrameric form (2 mM biotin or glycine buffer pH 2.0).

The combination was first developed to a peptide ligand, because photophores and biotin can be introduced separately at lysine α- and ε-amino groups (Gilbert and Rando 1995), but not all ligands have many modification sites in the ligand skeleton. One resolution is the synthesis of biotinylated photophore.

We described these methodologies to apply identifications of acceptor binding site of bovine β-1,4-galactosyltransferase (Gal T).

Glycosyltransferases, which had not been cleared functional analysis, were suitable examples to analysis with photoaffinity labeling. The asparagine moiety in *N*-acetyl glucosamine-asparagine skeleton, which is a acceptor substrate for β-1,4-galactosyltransferase (EC 2.4.1.38), was modified with biotinylated diazirinyl photophore, and the binding site in the protein was revealed for the first time (Fig. 1.3 and Table 1.1) (Hatanaka et al. 1996a, b; Hatanaka and Kanaoka 1998).

Our previous study on β-1,4-galactosyltransferase demonstrated that photoaffinity labeling can reveal conformational change during the enzymatic reaction. The enzyme has two substrates, UDP-galactose as a donor and *N*-acetyl-D-glucosamine (GlcNAc) as an acceptor to generate *N*-acetyllactosamine. The biotinylated photoreactive GlcNAc derivative **12** was prepared for this purpose. Specific photoincorporation of photoreactive GlcNAc derivatives was achieved only in the presence of UMP, which is a UDP-Gal analogue at 37 °C. The results indicated that the two substrates should be orientated near position in the active site to promote conformational change of the enzyme structure (Hashimoto and Hatanaka 1999; Hatanaka

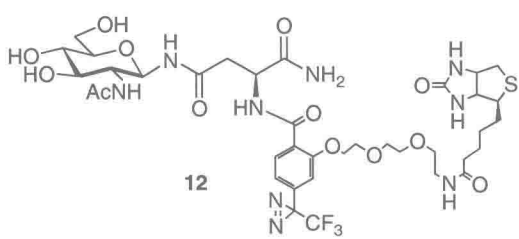


Fig. 1.3 Biotinylated photoreactive GlcNAc derivative for photoaffinity labeling of bovine β-1,4-galactosyltransferase

Table 1.1 Photoaffinity labeling yield of bovine GalT with compound **12** in various conditions

Compound **12**	+	+	+	+
UMP	–	+	+	+
Temperature (°C)	37	37	23	4
Specific labeling (%)	1.7	4.0	2.5	0.6

et al. 1998, 2001). It was also reported that dynamic structural investigations on the torpedo nicotinic acetylcholine receptor were performed by time-resolved photoaffinity labeling (Mourot et al. 2006).

The enzyme also utilized donor substrate UDP-Gal, which is common donor substrate for other galactosyltransferases. The kinetic study revealed that the photoreactive acceptor substrate analogue had enough affinity for target enzymes (observed Km were 0.17 and 0.06 mM for GlcNAc and 12, respectively). The photoaffinity labeling of GalT with compound **12** was set up, at ten times higher substrate concentration against Km value. The enzymatic reaction mixture was incubated at optimal temperature (37 °C) for 10 min to make complex with substrate, followed by irradiation at 4 °C. The sample was subjected to detect biotinylated proteins by avidin–biotin specific interaction with chemiluminescence. The chemiluminescence signals were quantified with external biotinylated proteins. The results show that both incubation at optimal temperature and the presence of donor substrate, UMP as inhibitor for UDP-Gal, were important to detect specific labeling. Photoaffinity labeling enables us to judge molecular dynamics (conformational change) under setting up labeling conditions.

Preparative scale of photoaffinity labeling for GalT was setting up from preliminary results, including UMP, which was one of the substrate mimics for GalT. Streptavidin–peroxidase conjugate was applied for photoaffinity label experiments, and immobilized streptavidin was utilized for isolation of photoaffinity-labeled components. The cell lysate overexpressed GalT in *E. coli* cell was applied to photolabeling with compound **12**. The results revealed the biotin components were introduced to GalT specifically in the cell lysate, even though the photolabeled efficiency of compound **12** and GalT was calculated less 4%.

Photoaffinity-labeled mixture was subjected to immobilized avidin to isolate photolabeled GalT specifically. The unlabeled GalT was passed through the affinity column and the labeled Galt was recovered with SDS sample buffer at 100 °C (for streptavidin) or excess biotin (for monomeric_avidin). The purified photolabeled GalT was subjected to enzymatic digestion, followed by HPLC analysis. The several peaks were detected and each fraction was submitted blotting on PVDF membrane to detect biotin components. But the small peptides did not retain PVDF membrane due to increase hydrophobicity. *p*-Nitrodiazirine was modified on PVDF membrane followed by irradiation with black light followed by reduction of nitro group to amine to form amide bond with carboxylic acid of peptides in the presence of carbodiimide. The biotin-based chemiluminescence was detected in one of the major peptides, and the sequence analysis revealed it as Y197-R208 peptide. Combination of molecular docking study and photoaffinity-labeled results strongly suggests that the carboxylate group of Asp318 could be involved in the activation of acceptor sugar 4-OH for the efficient galactosyltransfer (Fig. 1.4).

Although avidin–biotin interaction is very strong, and formation of the complex is very fast and rigid, it is too difficult to dissociate the biotin molecule from the complex as a native form. Very harsh conditions are needed for the biomolecules

0 MKFREPLLGG SAAMPGASLQ RACRLLVAVC ALHLGVTLVY YLAGRDLRRL PQLVGVHPPL

60 QGSSHGAAAI GQPSGELRLR GVAPPPPLQN SSKPRSRAPS NLDAYSHPGP GPGPGSNLTS

120 APVPSTTTRS LTACPEESPL LVGPMLIEFN IPVDLKLVEQ QNPKVKLGGR YTPMDCISPH

180 KVAIIIPFRN RQEHLK**YWLY YLHPILQR**QQ LDYGIYVINQ AGESMFNRAK LLNVGFKEAL

240 KDYDYNCFVF SDVDLIPMND HNTYRCFSQP RHISVAMDKF GFSLPYVQYF GGVSALSKQQ

300 FLSINGFPNN YWGWGGE**D**DD IYNRLAFRGM SVSRPNAVIG KCRMIRHSRD KKNEPNPQRF

360 DRIAHTKETM LSDGLNSLTY MVLEVQRYPL YTKITVDIGT PS

Fig. 1.4 β-1,4-Galactosyltransferase (bovine) sequence. *Bold underlined* sequence (Y197-R208) was photolabeled sequence with compound **12**. *Bold* at ^{318}D could play important roles for the activation of acceptor with docking study

(70% HCOOH at r.t., autoclave, or 2% SDS—8 M urea with boiling), and the recovery yield of the biotinylated biomolecule is not quantitative.

Since biotin–(strept)avidin binding is essentially irreversible, several approaches have been investigated to achieve the efficient recovery of biotinylated products from (strept)avidin-immobilized matrix.

Several approaches were examined for this problem. In one, modified avidin, which is described above, was used. Another introduced the chemical cleavable tags between the photophore and biotin to isolate photolabeled components from the avidin–biotin complex. Diol (Hashimoto et al. 2001) and disulfide moieties (Hashimoto et al. 2001) are well used for this purpose with isolation with thiol and periodate, respectively; however these moieties are also present in biomolecules (disulfide bond and sugar moiety), so it is not a specific method for labeled biomolecules. Periodate-treated diol, which generates aldehyde, can react with hydrazine as post-functional modification, and the detection limit of aldehyde and biotin hydrazide was estimated over 0.2 pmol (Hashimoto and Hatanaka 2005b).

It was reported that nitrophenyl (Olejnik et al. 1995; Fang et al. 1998; Fang and Bergstrom 2003) and sulfonamide moiety (Park et al. 2005) were suitable for the purpose. These moieties are less abundant in biomolecules and the cleavable condition is very mild in almost all biomolecules.

The final possibility is to utilize site-specific digestion enzymes for the ligand skeleton (Hashimoto et al. 2004). The protected amino acid residue was inserted between diazirine and biotin and was easily deprotected and then acted as a substrate for digestion enzymes. Folding biomolecules were not digested under this condition, although the introduced amino acid also became a substrate when the protecting groups were removed. Digestion was regulated by a methyl ester, which is a basic protecting group in organic chemistry. This combination was utilized in not only protein mixtures but also digested mixtures. The combination of the

glutamic acid γ-methyl ester and V8 protease revealed that effective retrieval of labeled components is possible. For labeled biocomponents, it is first enzymatically digested and then deprotected, followed by redigestion to cleave the avidin–biotin complex to isolate the labeled peptides. Furthermore, the combination of linker amino acid and digested enzyme had many patterns to facilitate handling of the labeled components.

1.5 PAL with Other Specific Interactions

Oligo acidic or basic amino acids could shift the isoelectric point of target proteins and shield with protecting groups, which are widely used in amino acid chemistry. The e-protected penta-Lys ([Lys(Boc)]$_5$) was neutralized, but acid treatment, which deprotected Boc group, caused the compound to have high basicity. The properties combined with photoaffinity labeling. The diazirine derivative was condensed with C-terminal of biotin-[Lys(Boc)]$_5$, and the reagents were subjected to chromatofocusing. Acid treatment promoted, which deprotected Boc group, the isoelectric point which changed the pH from 5 to 7 because all e-amino group was deprotected. It is very convenience that the protecting group can be easily distinguished from amino acid moiety from protein. The properties can apply to the enzymatic digestion. Lys in the peptides is recognized by endopeptidase Lys-C or trypsin. The synthetic compound without any treatment was resisted to the trypsin digestion, but was easily digested after deprotection with acid followed by trypsin treatment. The cleavable property caused the isoelectric point to return to the original value. The properties are also utilized in the photolabeled component that can change the isoelectric point to isolate the original protein and return the original isoelectric point after acid treatment followed by digestion to release penta-Lys moiety (Hashimoto and Hatanaka 2006).

1.6 Limitation

Although photoaffinity biotinylation was very useful to identify the labeled region, the large modification with the ligand skeleton sometimes causes decreased affinity of the synthetic compounds. For example, fatty acid derivatives, which contained diazirine photophore and biotin (Fig. 1.5a) (Hashimoto and Hatanaka 2005a, b), cannot be utilized for photoaffinity labeling of sphingolipid ceramide *N*-deacylase (SCDase). Biotin and photophore have to be introduced at different part of ceramide to recognized by the enzyme (Fig. 1.5b) (Hashimoto and Hatanaka 2008b). The limitation promoted the development of post-modification of labeled components, which is summarized in the next chapter.

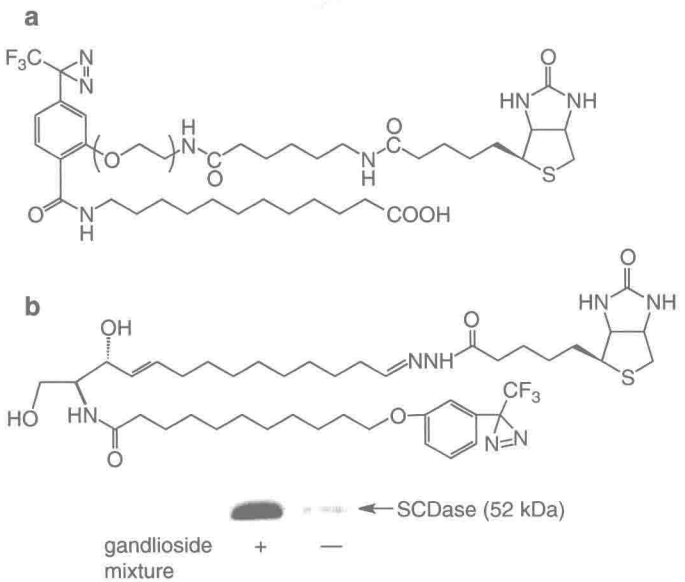


Fig. 1.5 Photoaffinity biotinylation probes for sphingolipid ceramide *N*-deacylase (SCDase). (**a**) Photoaffinity biotinylation fatty acid derivative, which was not recognized by SCDase. (**b**) Photoaffinity biotinylation ceramide derivative, which was recognized by SCDase

1.7 Conclusion

The combinations of photoaffinity labeling and avidin–biotin systems will be developed not only to identify and to detect photolabeled biomolecules but also to identify photolabeled regions. The low detection limits for avidin–biotin systems are very useful, but irreversible dissociations make some troubles to isolation of labeled components. Many efforts to overcome these problems, which are based on chemical structure of ligand derivatives and modified avidin to dissociate effectively, have been made over the last two decades.

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