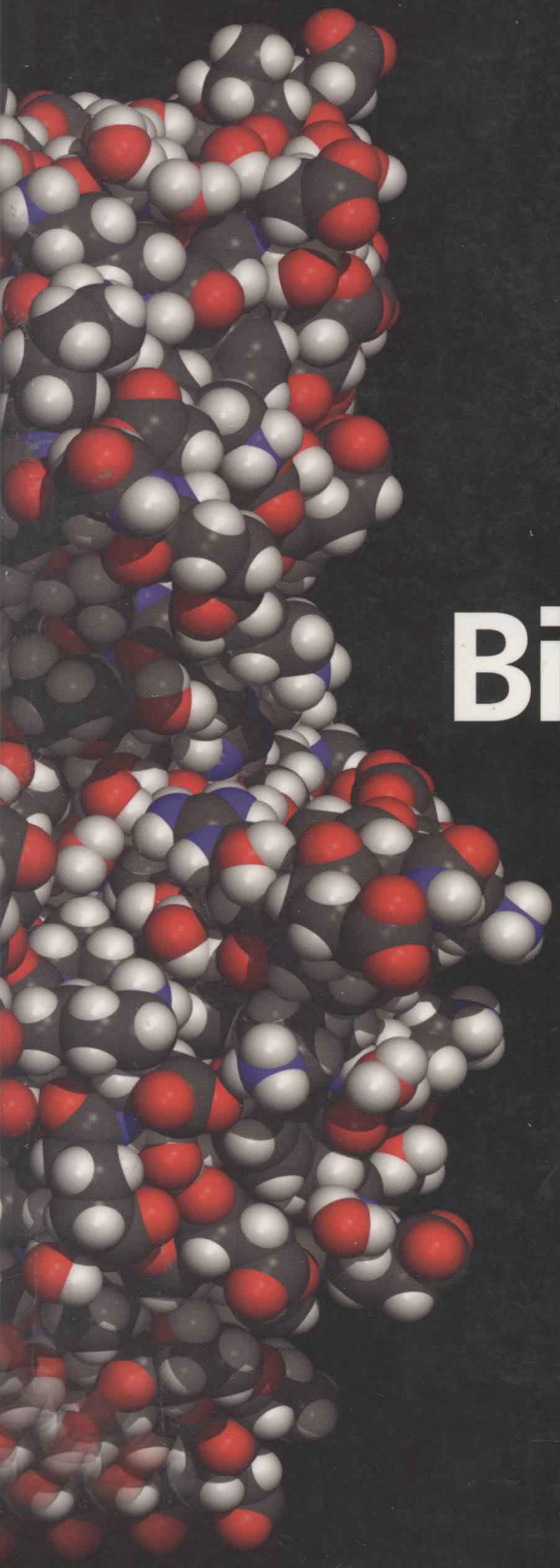

Second Edition

Bioconjugate Techniques

Greg T. Hermanson

Bioconjugate Techniques

2nd Edition

Greg T. Hermanson

Pierce Biotechnology,
Thermo Fisher Scientific,
Rockford, Illinois, USA

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The molecular model on the cover is a nitrite reductase enzyme obtained from the RCSB Protein Data Bank (2afn), as determined by Murphy, M.E., Turley, S., Kukimoto, M., Nishiyama, M., Horinouchi, S., Sasaki, H., Tanokura, M., and Adman, E.T. (1995) Structure of Alcaligenes faecalis nitrite reductase and a copper site mutant, M150E, that contains zinc. Biochemistry 34, 12107–12117. The space-filling model was created from the coordinate file using PovChem and the final image ray-traced using POV-Ray

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2nd Edition

For Amy and Meghan, who, since the first edition was published, have now become interested in pursuing careers in microbiology and medicine.

Preface to the Second Edition

In the decade since the publication of the first edition, the field of bioconjugation has advanced at an incredible pace. Tens of thousands of additional publications have appeared in the biological, medical, polymer, material science, and chemistry journals describing novel reactions and reagents along with their use in a variety of bioconjugate techniques. In some cases, the innovative application of relatively old organic reactions that now are used to solve new bioconjugation problems has resulted in significant advances in the field. Today, there are more options available than ever before to create nearly any covalent complex imaginable between molecules of virtually any type. In addition, exciting new methods of detecting biomolecules and their interactions have been made possible by recent inventions in bioconjugate chemistry.

Many of the new reactions, reagents, and applications that are featured in this edition didn't even exist at the time that the previous edition was written. For instance, although the preparation of inorganic quantum dots had been described in the physics and material science literature at the time that the first edition was published, their luminescent properties were not applied to biomolecule labeling until only recently. Similarly, the benefits of short hydrophilic polyethylene glycol (PEG)-based spacers in the creation of bioconjugate reagents were mentioned in the first edition, but only within the last few years has a broad range of crosslinkers and modification reagents become available which take advantage of their characteristics.

The recent advances in bioconjugation have resulted in major new sections in this edition, including chapters on Dendrimers and Dendrons; Silane Coupling Agents; Microparticles and Nanoparticles; Buckyballs, Fullerenes, and Carbon Nanotubes; Mass Tags and Isotope Tags; Chemoselective Ligation and Bioorthogonal Reagents; Discrete PEG Compounds; and a chapter on Bioconjugation for the Study of Protein Interactions. In addition, many of the previous chapters now include important additions that include highlights of new reactions and reagents, which reflect the major inventions and innovations made in the field in recent years. For instance, the chapter on Fluorescent Probes now has three new sections: Cyanine Dye Derivatives, Lanthanide Chelates for Time Resolved Fluorescence, and Quantum Dot Nanocrystals. There also are new sections describing protein oxidation reactions, solvent accessibility of functional groups within proteins, and the latest information related to the modification of glycans and other carbohydrates. Many new reagents also are described throughout the updated chapters that were a part of the first edition of the book.

With these new additions comes nearly a doubling of the number of key references cited along with a considerable amount of citation updates throughout the original material. However, the references cited within the book are not designed by any means to be exhaustive for each topic, but rather are intended to provide good starting points for understanding the

concepts and obtaining additional information as needed. For this reason, many review articles are cited along with the first publications describing new reagents or new techniques.

A significant aid in the preparation of the second edition was the tremendous resources now available on the Internet for searching references to virtually any subject or key word within the scientific literature. For this reason, adding endless references to each chapter probably only would increase the size of the book by hundreds of pages, but add very little real value. Far better is for the reader to make use of pertinent Internet databases to search for key words, structure names, or reagent acronyms which can provide lists of hundreds or even thousands of additional references or links regarding any bioconjugation technique of interest.

Some recommended Internet resources for finding bioconjugation-related information include the general Internet search engines like Google or Yahoo in order to obtain a broad spectrum of hits to any bioconjugation topic. This type of search will yield publications, valuable information on web sites dedicated to the desired subject matter, and possible commercial sources for particular reagents. Google Scholar (http://scholar.google.com/) is especially good at finding a broad selection of hits to key words or authors in any field, although its inability to sort the results makes it somewhat limited. In addition, several other dedicated reference databases for science-related topics can be used to complement these general search engines and provide a full spectrum of topical references.

Some Internet search sites that I have found particularly useful include the National Center for Biotechnology information (NCBI) Entrez cross-database search page (http://www.ncbi.nlm.nih.gov/sites/gquery), which includes PubMed Central containing a limited number of free, full text journal articles. In addition, HighWire Press run by Stanford University also contains many free articles from established journals (http://highwire.stanford.edu/) and is able to search the PubMed database simultaneously.

However, some bioconjugation references can't be found in these databases. Some key word searches would yield many additional hits within the chemistry or physical science journals than a search restricted exclusively to the life science journals. For searching within both the life science and physical science journals, perhaps the best option is a multi-database search engine, such as Scirus (http://www.scirus.com). This site is able to search for key words in over 450 million web pages, including all the major science journals. The combined database search can yield many bioconjugation-related references unavailable on the other life science-specific portals. For instance, a search for "dendrimer" on the Entrez cross-database search page returns 1,187 PubMed citations, whereas a Scirus search provides 3,979 hits. The difference relates to the journals that are covered in the database search engine, and the Scirus site accesses the chemistry, polymer, and physics journals inaccessible through PubMed. In addition, Scirus allows searches of any mention of key words on university or institute web pages as well as any other web sites mentioning the specific topic. Including these other sources for a search of "dendrimer" returns a total of 27,708 hits.

Finally, journal web sites and fee-based services can be used with success to find additional references to key topics. Examples of services that are particularly good include the American Chemical Society's Chemical Abstracts Service (CAS; http://www.cas.org/) and their journal search page (http://pubs.acs.org/index.html); the Elsevier Scopus search engine (http://info.scopus.com/) and ScienceDirect database (http://www.sciencedirect.com/); and the ISI Web of Knowledge (http://isiwebofknowledge.com/).

The published procedures that can be found in the journal articles, books, academic web pages, and commercial instruction manuals for particular reagents all formed the basis for

most of the protocols described in this edition. These general methods should be used as starting points for optimizing each conjugation process for a unique application. Often when working with biological molecules like proteins, a method optimized for one protein may need to be adjusted to take into consideration the unique properties of another protein. For instance, it may be simple to conjugate or modify highly soluble proteins that have a high degree of conformational stability. However, similar reactions done on hydrophobic membrane proteins or insoluble peptide sequences often will require changes to the reaction conditions to effect the same conjugation process.

It is my hope that this second edition of Bioconjugate Techniques may stimulate even more ideas, inventions, and innovations and prove useful to scientists in every field who want to take advantage of bioconjugation to create novel tools for research, diagnostics, and therapeutics.

Preface to the First Edition

Bioconjugation involves the linking of two or more molecules to form a novel complex having the combined properties of its individual components. Natural or synthetic compounds with their individual activities can be chemically combined to create unique substances possessing carefully engineered characteristics. Thus, a protein able to bind discretely to a target molecule within a complex mixture may be crosslinked with another molecule capable of being detected to form a traceable conjugate. The detection component provides visibility for the targeting component, producing a complex that can be localized, followed through various processes, or used for measurement.

The technology of bioconjugation has affected nearly every discipline in the life sciences. The application of the available crosslinking reactions and reagent systems for creating novel conjugates with peculiar activities has made possible the assay of minute quantities of substances, the *in vivo* targeting of molecules, and the modulation of specific biological processes. Modified or conjugated molecules have been used for purification, for detection or localization of specific cellular components, and in the treatment of disease.

The ability to chemically attach one molecule to another has caused the birth of billion-dollar industries serving research, diagnostics, and therapeutic markets. A significant portion of all biological assays, including clinical testing, is now done using unique conjugates that have the ability to interact with particular analytes in solutions, cells, or tissues. Crosslinking and modifying agents can be applied to alter the native state and function of peptides and proteins, sugars and polysaccharides, nucleic acids and oligonucleotides, lipids, and almost any other imaginable molecule that can be chemically derivatized. Through careful modification or conjugation strategies, the structure and function of proteins can be investigated, active site conformation discovered, or receptor–ligand interactions revealed. Without the development of bioconjugate chemistry to produce the associated labeled, modified, or conjugated molecules, much of life science research as we know it today would be impossible.

Bioconjugate Techniques attempts to capture the essence of this field through three main sections: its chemistry, reagent systems, and principal applications. Although the scope of bioconjugate technology is enormous, this book provides for the first time a practical overview that condenses this breadth into a single volume. Part I, Bioconjugate Chemistry, begins with a review of the major chemical groups on target molecules that can be used in modification or crosslinking reactions. The chemical reactivities and native properties of proteins, carbohydrates, and nucleic acids are examined in separate chapters, with a view toward designing conjugation strategies that work. Next is a discussion on how to create particular functional groups on these molecules where none exist, or how to transform one chemical group into another. Blocking agents also are examined in this section. The last chapter in Part I summarizes all the major reactions used in bioconjugate chemistry in brief, easy-to-follow descriptions, with liberal references to the literature and to other parts of the book where the reactions are put to use.

Part II, Bioconjugate Reagents, provides a detailed overview organized both by reagent type and by chemical reactivity to present all the major modification and conjugation chemicals commonly used today. The first section in this part examines true crosslinking agents. Zero-length crosslinkers, homobifunctional and heterobi-functional crosslinking agents, and the new trifunctional reagents are discussed with regard to their reactivities, physical properties, and commercial availability. In many cases, conjugation strategies and suggested protocols are presented to illustrate how the reagents may be used in real applications. The next section, Tags and Probes, discusses modification reagents capable of adding fluorescent, radioactive, or biotin labels to molecules. Major fluorophores, including fluorescein, rhodamine, and coumarin derivatives as well as many others, are presented with modification protocols for attaching them to proteins and other molecules. In addition, procedures and compounds for adding radiolabels to molecules, including iodination reagents for ^{125}I-labeling and bifunctional chelating agents to facilitate labeling with other radioisotopes, are discussed. Finally, numerous biotinylation reagents are presented along with protocols for adding a biotin handle to macromolecules for subsequent detection using avidin or streptavidin conjugates.

Part III is by far the largest portion of the book. Bioconjugate Applications discusses how to prepare unique conjugates and labeled molecules for use in particular application areas. This includes: (1) preparing hapten–carrier conjugates for immunization, antibody production, or vaccine research; (2) manufacturing antibody–enzyme conjugates for use in enzyme immunoassay systems; (3) preparing antibody–toxin conjugates for use as targeted therapeutic agents; (4) making lipid and liposome conjugates and derivatives; (5) producing conjugates of avidin or streptavidin for use in avidin–biotin assays; (6) labeling molecules with colloidal gold for sensitive detection purposes; (7) producing polymer conjugates with PEG or dextran to modulate bioactivity or stability of macromolecules; (8) enzyme modification and conjugation strategies; and (9) nucleic acid and oligonucleotide conjugation techniques.

Each of these application areas involves cutting-edge technologies that rely heavily on bioconjugate techniques. In many cases, without the basic ability to attach one molecule to another much of the research progress in these fields would grind to a halt. Bioconjugation thus is not the end but the means to providing the reagent tools necessary to do other research or to produce assays, detection systems, or therapeutic agents.

The purpose of this book is to capture this field in an understandable and practical way, providing the foundation and techniques required to design and synthesize any bioconjugate desired. To aid in this process, over 1,100 pertinent references are cited and over 650 illustrations depicting reactions and chemical compounds are presented. Hundreds of bioconjugate reagents are examined for use in dozens and dozens of potential applications.

The choices available for producing any one conjugate can be overwhelming. I have attempted to identify the best reagents for use in particular application areas, but the presentation is by no means exhaustive. In addition, most of the protocols included in the book are generalized or based on personal experience or literature citations directed at particular applications. Occasionally, applying a bioconjugate protocol that works well in one instance to another application may not work as expected. One or more of the components of the conjugate may lose activity, the conjugate may precipitate, or yields may not be acceptable. In almost every case, some optimization of reaction conditions of reagent choices will have to be done to produce the best possible conjugate or modified molecule for use in a new application. Even protocols as common as antibody–enzyme conjugation techniques may need to be altered somewhat for each new antibody complex produced. The best strategy is to use the suggested protocols, literature citations, and insights gained from this book as starting points to create a bioconjugate that will work well in your own unique application.

Greg T. Hermanson

Acknowledgments

I again acknowledge the large number of scientists who made valuable contributions to the field of bioconjugation in general and to the contents of this book in particular. First, thanks to the thousands of researchers, many of whose names appear in the reference section, which developed and optimized the hundreds of reagents and applications related to the modification and conjugation of biomolecules. I also want to thank Barb Tanaglia, Sally Etheridge, Crystal Gomez, and Heather Flynn for their expert help in obtaining journal references and directing me to the best Internet databases useful for searching the scientific literature. I also thank Craig Smith for reviewing the new material and being so supportive of my writing. Finally, I want to acknowledge David Dellapa, Tom Currier, and Alan Doernberg for giving me corporate approval for this entire endeavor. Although Thermo Fisher Scientific did not sponsor the project, the company provided great motivation for me to undertake the effort and complete the second edition.

Finally, special thanks to the one who made it all possible.

Health and Safety

This book describes hundreds of reagents, reactions, and applications for use in bioconjugation. Most of the compounds are highly specialized and have very little information regarding their toxicological properties. However, the overwhelming majority is known to be reactive and can be corrosive, hazardous, toxic, or dangerous to personal health and safety. At a minimum, bioconjugation reagents should be considered irritants and handled with care. In addition, the disposal of excess reagents or reaction by-products could be harmful to the environment. For this reason, the use of any reagent or protocol described in this book should be done with caution. Refer to the appropriate Material Safety Data Sheet (MSDS) for every compound or component used in a reaction before starting an experiment. The use of personal protective equipment, fume hoods, and proper laboratory techniques can assure safety for both the user and other people in the immediate area.

In addition, the disposal of waste materials should be done according to the appropriate environmental regulations to prevent toxic elements or compounds from entering the water, air, or soil.

It is best to consider every reaction as potentially dangerous and every compound as potentially hazardous (or at least a strong irritant) to avoid injury or damage to health.

Intellectual Property

Throughout this book I have tried to provide the key references to reagents, reactions, and techniques used in bioconjugation. However, such knowledge does not necessarily provide the freedom to legally use them without consideration for existing intellectual property rights. While in some cases, pertinent patent references are provided within the book, this is done only to supply additional technical details about the topic being discussed.

Today, nearly every important reagent or method reported in the literature has a patent or patent application associated with it, especially if it has potential commercial value. A search of the patent databases, such as the United States Patent and Trademark Office (http://www.uspto.gov/) or the European Patent Office (http://ep.espacenet.com/) for key words or the potential names of inventors can provide a list of any existing issued patents or patent applications related to a bioconjugate technique or compound. In addition, a fee-based service such as Delphion is particularly effective at finding patents related to any subject matter (http://www.delphion.com/).

It is the responsibility of the reader to become familiar with patents that may cover particular compounds, compositions, reactions, or their use in bioconjugation applications. If patents or patent applications exist, it is important that permission or a license be obtained to use it before exploiting any intellectual property for commercial use.

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