New Science Through Assemblies of Scientists and Molecules

David G. Whitten, NSF Center for Photoinduced Charge Transfer and Department of Chemistry, University of Rochester, Rochester, New York 14627

The purpose of this article is to discuss some current work in which the emphasis is on uncovering and developing new reactivity in assemblies or aggregates of molecules. While the discussion will try to point out some of the interesting results that have been obtained in a number of cases, it will also touch to some extent on the "sociology" of science in which nonobjective influences such as "fashion", disciplinary boundaries and even words or names may play a much greater role than many scientists would like to admit. One recognizes from the outset that scientists and certainly chemists from different disciplines can look at the same set of experimental results from quite different perspectives. Thus an organic chemist interested in the clean synthesis of a relatively small, soluble molecule can be dismayed by the production of a dark insoluble residue as the outcome of an attempted reaction. The same product might, on the other hand, delight a polymer or colloid scientist seeking to generate insoluble macromolecules or macromolecular assemblies. Frequently people working in different disciplines may even have different terminology to discuss common phenomena, a complication which can impede interdisciplinary discussions and certainly hinder collaboration. Over the past five years the NSF Center for Photoinduced Charge Transfer in Rochester has made possible a new collaboration between industrial and university scientists in an active partnership which, while centered in chemistry and in particular in photochemistry, involves scientists from several disciplines including physics, chemical engineering, optics and biology. In this article we will discuss one area of investigation and illustrate how the collaboration has enabled progress that would otherwise have been very difficult to achieve.

Scientists working with dyes and pigments have long recognized that association and/or aggregation effects play a large role in the color, physical properties such as solubility and photophysical behavior.\textsuperscript{1,2} Two limiting types of aggregate that have been defined are J- and H-aggregates for a variety of dyes, especially cyanines. J-aggregates are characterized by a relatively large red shift in the lowest absorption transition relative to nonaggregated dye while H-aggregates are those having a corresponding blue shift in the lowest energy transition compared to monomer. Although the importance of these aggregates—especially J-aggregates—in specific areas such as the photophysical steps in dye sensitization in silver halide photography has been long recognized, and several attempts have been made at modeling them both experimentally with simple well-defined systems and theoretically,\textsuperscript{1-9} their occasional encounter by organic chemists working with relatively large molecules in solution as insoluble residues or as unexpected and often uncharacterizable products has led mostly to frustration and, until recently, to few attempts to develop any specific structure-reactivity relationships or even to understand the factors that control aggregate formation in solution or microheterogeneous media.

However in recent years the surge of interest in "nanoparticles", mostly of inorganic substances such as colloidal semiconductors, metal clusters, etc., and the nearly simultaneous development of new areas of "host-guest" phenomena and "supramolecular" chemistry\textsuperscript{10} has led to a recognition that organic molecules may be a particularly rich source of important

Continued on page 2
new materials with interesting ground and excited state properties in assemblies produced by the controlled association of unsaturated compounds, aromatics or functionalized aromatic molecules.

Involvement of our laboratory in the chemistry and photochemistry of organic aggregates began several years ago when we initiated some studies on reactivity in organized assemblies such as micelles, bilayer vesicles and Langmuir-Blodgett (LB) assemblies. Since LB films of pure fatty acids are essentially insulating and of thickness 25-30 angstroms, we were interested in the effects that incorporation of a conjugated system into the film might provide, especially in enhancing energy transfer or electron transfer across the film. In our studies we constructed a number of "modified" fatty acids containing a photochemically reactive or spectroscopically active chromophore. Although our usual idea was to incorporate the functionalized "guest" or solute in the fatty acid host with the latter as an effective solute, we realized that the solute/solvent mole ratio was exceedingly high in these cases compared with the organic solutions we were more accustomed to investigate, and very high effective solute concentrations would be present. Not surprisingly, in a number of instances we found that the film-incorporated functionalized fatty acid showed different properties from the same chromophore incorporated into a dilute organic solution. One of the first cases to indicate rather clear effects due to aggregate formation was the Langmuir-Blodgett films and assemblies formed from trans-stilbene functionalized fatty acid derivatives of general structure 1 (SFA).\textsuperscript{11-14}

\[
\text{CH}_3(\text{CH}_2)_5 - \begin{array}{c}\text{H} \\
\text{H}
\end{array} - \begin{array}{c}\text{H} \\
\text{H}
\end{array} - (\text{CH}_2)_3\text{COOH}
\]

\[n \text{S}_m \text{A} = \varepsilon \text{S}_4 \text{A}
\]

We found that films of the SFAs, either pure or diluted with saturated fatty acids of the same overall length, for extended conformations exhibited absorption and emission spectra quite different from those of the monomer in organic solution. The prominent absorption transition is blue shifted by some 70 nm while the fluorescence shows a red shift and a very prominent component of relatively long wavelength.\textsuperscript{11} That the substantive spectral changes observed were due to a physical phenomenon rather than to any chemical reaction was shown by the fact that we could nearly quantitatively recover the monomer simply by dissolving the spread film or transferred assembly in an organic solvent. Interestingly we found that even under substantial dilution of the SFAs in Langmuir-Blodgett films the H-aggregate was the chief species detectable by both absorption and fluorescence.\textsuperscript{11,13} In subsequent investigations we have found that formation of H-aggregates by a large number of SFAs, substituted-SFAs and higher vinyllogues such as diphenylbutadiene and diphenylhexatriene derivatives is a quite general process in both Langmuir-Blodgett assemblies and in other organized media such as bilayer vesicles.\textsuperscript{13,15} Using some simple modeling techniques available to us at the time of these investigations, we rationalized the formation of these aggregates in terms of a head-to-head packing of the trans-stilbene chromophores which could be imposed as the films were compressed at the air-water interface; calculations predicted a forbidden low energy transition and an allowed high energy transition and a limiting aggregate size which could be reached when the number of molecules reached approximately six.\textsuperscript{11} Our failure to eliminate the aggregate by dilution was attributed to "imperfect behavior" of the surfactant mixture in the spreading process or to "microcrystal formation." In our studies of photoinduced energy transfer and electron transfer processes, we observed that the aggregated SFAs were quenched efficiently by electron acceptors within the same layer or by singlet energy acceptors over relatively long distances; however, degenerate singlet energy transfer between SFAs in different monolayers in a multilayer assembly was very ineffective due to the low oscillator strength of the aggregated SFA.\textsuperscript{12} Thus this early encounter with aggregate formation in LB assemblies was mostly regarded as a partially explainable nuisance.

About the same time as our investigation of SFAs in Langmuir-Blodgett assemblies was going on, we were also examining some substituted meso-tetraphenylporphyrin derivatives, examples of the so-called "picket fence" por-
Phyrins initially developed by Collman and coworkers for the development of a number of heme enzyme mimics. Compounds of the type 2a-d were of interest to us, in part because we thought that the 4,0 atropisomer (2a) might incorporate well in an oriented manner into aqueous surfactant media such as detergent micelles and phospholipid vesicles. Although a few of these notions turned out to be well-founded, our initial experiments with a few of the series of 2a atropisomers in detergents such as sodium dodecylsulfate (SDS) led once again to peculiar, if also interesting, results. Most of the porphyrins of general structure 2 are well solubilized by suspending in aqueous detergent above the critical micelle concentration (cmc, > 50 mM), detergent concentration above which most of the detergent exists as micelle "droplets") to give what are clearly solutions of monomeric porphyrin which is almost entirely solubilized within (or on the surface) of the micelle. The porphyrins can be suspended in water without detergent but there is no true solubility without detergent. For most of the porphyrins we noted that addition of very small amounts of detergent results in a slow solubilization of the porphyrin which does not really begin until the detergent concentration is near or in excess of the critical micelle concentration. However, we were surprised to note that addition of detergent (~ 6 mM) to suspensions of 2a (specifically for the cases where R = C₆ or C₇) results in some clear solubilization to produce a new species which is characterized by a sharp main transition (Soret Band) which is shifted 15-20 nm to longer wavelengths. As more detergent is added this new transition is replaced by the "normal" transition associated with solubilized monomeric porphyrin. While this type of solubilization was observed for free base porphyrins and some metal complexes (Zn²⁺ and Pd²⁺), it was found exclusively for the 4,0 atropisomers (2a) and the specific chain lengths noted; there is also a specificity of chain length for the detergent (C₁₀-C₁₄). At the time these initial observations were made, we were intrigued by the results but we also had considerable difficulty in fully "solving" the nature or structure of the type of species we were dealing with in this "special solubilization" process. The clear restrictions for the atropisomer having one face "free", and the match between surfactant and porphyrin structure, suggested a complex with some sort of clear topological requirement. The photophysical properties of these surfactant-porphyrin complexes were also somewhat of a puzzle, since the sharp red-shifted Soret band suggested there should be strong fluorescence, but only relatively low fluorescence efficiency and low triplet yields were indicated. The restriction of complex formation to the 4,0 atropisomer suggested that selective photoatropisomerization of other isomers to the 4,0 followed by surfactant complex formation might provide a new path for selectively making this otherwise somewhat difficult-to-isolate material. However, experiments to test this proved
only marginally successful. The continuing interest and perseverance of two graduate students working on this problem, assisted greatly by consultation with and experimental assistance from some of our industrial "neighbors" at Kodak, convinced us that we were dealing with an unusual yet rather interesting case of "J" aggregation in which the surfactant-porphyrin association below the cmc was leading to an "offset" association of the porphyrin chromophores in accord with the predictions of geometric arrangements for J- and H-aggregates shown in Figure 1.\textsuperscript{9,11} Indeed these complexes proved to be one of very few examples of "J" type for porphyrins since the more commonly

![Diagram showing geometric arrangements of chromophores producing H- and J-aggregates and exciton band splitting.](image)

Figure 1: Geometric Arrangements of Chromophores Producing H- and J-Aggregates and Exciton Band Splitting

studied porphyrin dimers or other aggregates in which face-to-face alignment is enforced invariably show blue-shifted absorption as indicated in the figure. Careful evaluation of the stoichiometric requirements for complex formation and some of the properties of the incorporated porphyrin (free base basicity, etc.) led us to propose an extended structure as shown in Figure 2.\textsuperscript{17} We are currently using the information obtained from these investigations to formulate new solution aggregates which can be selectively formed due to molecular topological control.

![Diagram showing stoichiometry for a unit tetramer.](image)

\begin{align*}
\text{Stoichiometry for a Unit Tetramer:} \\
\# \text{ of } H_2P &= 4 \\
\# \text{ of } SDS^4 &= 12 \\
(H_2P)_4(SDS)_{12} \quad &\text{\footnote{Fully Included + \# Shared = 8 + 8/2 = 12}}
\end{align*}

Figure 2: Proposed Structure of Premicellar 4.0 THex-SDS J-aggregates

In more recent studies we have turned our attention to selective control of aggregate structure and properties by a combination of molecular structure and medium. One series of molecules we have been examining is functional-
ized squaraine dyes of general structure 3. These dyes are widely used in electrophotography as active agents in the photogeneration process. Upon excitation in solid—usually microcrystalline—films, the squaraine dyes, invariably aggregated, undergo effective electron-hole separation processes which can culminate in charge annihilation.\textsuperscript{18} In a study ongoing at the Center for Photoinduced Charge Transfer involving collaboration between Xerox, University of Rochester and Kodak scientists, we are examining both the aggregation process and the photophysical behavior of the aggregates formed. The squaraines are particularly interesting in that we find two distinct aggregates formed for a number of surfactant and amphiphilic derivatives. In most cases direct formation of LB assemblies by transfer of the spread film from the air-water interface to a rigid support leads to the formation of a species which shows prominent absorption near 530 nm, about 100 nm to the blue of the monomer in solution. For several compounds, heating of the LB films results in the clean conversion of this species to a new aggregate having a sharp transition to the red of the monomer. While the monomer in solution is strongly fluorescent, we have found that neither of the species generated in the LB assemblies are emissive for the several squaraines we have studied to date. We attribute the species produced in the LB assemblies to aggregates having different structures, possibly simple "H" and "J" species oriented as suggested in Figure 1. As with the SFAs described above, we find that the aggregates generated are formed by physical association, since the squaraine monomer is easily recovered simply by dissolving the LB assembly. Although the LB films show no emission, we do find that several of the blue-shifted species are fairly efficient at generating photocurrents upon irradiation of LB films on an optically transparent electrode.\textsuperscript{19} The photoelectrochemical effects we observe can be attributed to a photoinitiated electron-hole separation and subsequent events in which the aggregated squaraine functions as a photocatalyst. Interestingly, studies with quencher indicate a fairly long diffusion length for the exciton generated by photoexcitation. In very recent work we have been able to synthesize functionalized squaraines which form directly the red shifted form as well as compounds exhibiting reversible interconversion of "H" and "J" structures. We are currently examining host-guest complexes of several of the same squaraines and have obtained what we believe are "H" dimers in very recent experiments.

In other studies we have focused on a more detailed examination of the aggregation of the SFAs and related compounds.\textsuperscript{13} From the diagram in Figure 1 it seemed possible that assemblies of mixtures of SFAs with the stilbene chromophores in different positions on the fatty acid chain might lead to aggregates ranging from the simple H-aggregates already encountered to species approaching J-aggregates for SFAs with a large offset between nearest neighbors. In fact we have found that LB assemblies of mixtures of 3-5 SFAs of quite different structure show very similar absorption and fluorescence, indicating a preponderance of H-aggregate. However, the LB assemblies formed from the mixtures show very efficient interlayer singlet energy migration and make possible fairly long-range quenching of multilayer assemblies by electron acceptors and energy acceptors, in contrast to the results described above with assemblies constructed with single SFAs. In subsequent studies in the Center we have found that H-aggregate formation is a very general process for a wide variety of $\alpha,\omega$-diphenylpolyenes, including some surfactant derivatives substituted with strong donor and acceptor substituents and, perhaps more interestingly, for binary and even more complex mixtures.\textsuperscript{20} We have been able to demonstrate that a "self-sorting" process occurs at the air-water interface and during compression, leading to zones of largely pure aggregate of a single species. Interestingly we find that the H-aggregate is clearly an energy minimum in aqueous environments, probably favored by apolar association such has been observed for a variety of aromatics in other recent investigations. Current studies are employing SFA-substituted phospholipids of general structure 4, in which the two SFAs are either the same or
different structures and thus should be forced to have structures ranging from "H" to "J" dimers when incorporated into an ordered assembly such as a lipid bilayer.\textsuperscript{15} Our findings to date are that liposomes or vesicles formed from pure SFA phospholipids of 4 exist as H-aggregates above and below the bilayer phase transition from gel to liquid crystal. Photophysical experiments suggest that these strongly fluorescent H-aggregates may be characterized by a much less delocalized exciton than the squaraine aggregates discussed above.

Although our studies of the relation between monomer structure and aggregation type, structure and properties in different media are far from complete, the combined expertise of the different scientists collaborating through the Center has made possible relatively rapid progress in addressing this problem. It seems reasonable to anticipate a number of important applications for the materials and their unique properties that can result from controlled association of photoreactive organic units.

Acknowledgments

The work described in this article has been supported by the National Science Foundation Center for Photoinduced Charge Transfer (CHE-9120001) and by a research grant (CHE 9211586). Current co-workers at the University of Rochester include Cristina Geiger, Susan P. Spooner, Inna Furman, Xuedong Song, Baowen Zhang, Wanda Richard, Kangning Liang, Huijuan Chen. The industrial partners in the research are Kock Yee Law from Xerox Corporation and Thomas Penner and Avi Ulman from Eastman Kodak.

References


About the Author

David G. Whitten received his undergraduate and Ph.D. degrees from The Johns Hopkins University. He is the C.E. Kenneth Mees Professor of Chemistry and the Director of the NSF Science and Technology Center at the University of Rochester.

---

A Note From D.C. Neckers, Executive Director, Center for Photochemical Sciences

As those of us from the Center have traveled around various parts of the world, we’ve heard from many of our colleagues about articles in The Spectrum. We’re delighted with how often we hear from the readership. It’s gratifying to know that The Spectrum is being widely read by its audience.

My contribution From the Executive Director often generates comments, too. I intend them to be provocative. On occasion my opinions may rangle in the greater community. To the extent it doesn’t become excessive, we’ll sometimes publish rebuttals.

That’s the situation this time. In an editorial several issues ago, I made a point about “changes in science” over recent years. The point was that, for example, molecular biology—which no one had heard of 25 years ago—was now a major scientific endeavor, while nuclear physics—which captured the brightest of the generations during and immediately after World War II—was doing substantially less so now.

My friends in the nuclear physics community begged to disagree. Therefore we are publishing excerpts of their letter (page 27).

In this issue of The Spectrum we are publishing five articles instead of the usual four. I hope, on behalf of the entire Center for Photochemical Sciences, that you enjoy this expanded version. I look forward to having more space in the next issue for my regular From the Executive Director column.

In the meantime, best wishes for the fall.

---

In This Issue

New Science Through Assemblies of Scientists and Molecules ........................................... 1

Photochemical Neutralization of HIV-1 and Inhibition of HIV-1-Induced Syncytium Formation
by Halogenated 1,8-Naphthalimides ................................................................. 8

Applications of Enhanced Chemiluminescence from Enzyme-Triggerable 1,2-Dioxetanes .......... 15

Picosecond Transient Raman Studies of Polyene Photosomerization .................................. 20

Present and Future Uses of Phototherapy in Medicine .................................................... 28
Photochemical Neutralization of HIV-1 and Inhibition of HIV-1–Induced Syncytium Formation by Halogenated 1,8-Naphthalimides

David E. Lewis and Ronald E. Utecht, Department of Chemistry, South Dakota State University, Brookings, SD 57007; Millard M. Judy and James L. Matthews, Baylor Research Institute, Dallas, TX 75228; Tran C. Chanh, Department of Virology and Immunology and Center for AIDS Research, Southwest Foundation for Biomedical Research, San Antonio, TX 78228

Introduction

The traditional methods for eradication of tumor cells or viruses frequently target the tumor-specific or virus-specific components of the target system (e.g. the HIV protease). In recent years, methods for neutralizing pathogens and tumors by using photochemistry based on membrane-bound lipophilic dyes as mediators of a phototoxic effect have been the subject of intense study. Thus, visible light has been used to effect cell kill in tumors or in disorders such as psoriasis by using a membrane-bound dye (typically a porphyrin or other polypyrrolemethine, a cyanine or a psoralen) to mediate the photochemical inactivation process. Where the photosensitizer is a porphyrin or a cyanine, it is generally accepted that the dye photosensitizes oxygen within the membrane, and that cell death is then due to oxidative degradation of membrane components by singlet oxygen or other peroxidic photoproducts. Cell kill due to sensitization of oxygen is usually termed the photodynamic effect, and its clinical application is photodynamic therapy. The psoralens act by a different mechanism which involves direct, oxygen-independent photochemical modification of the nucleic acid.

Enveloped viruses are characterized by the presence of a lipid bilayer envelope derived from the host cell which encapsulates the viral capsid. Because of the presence of this membrane, it has been a logical extrapolation of the work with cells to attempt photodynamic inactivation of enveloped viruses. In recent years, the application of the photodynamic effect has, indeed, been extended to include inactivation of enveloped viruses, and the porphyrins and cyanines have proved to be especially effective at inactivating these pathogens in whole blood.

In contrast to the classical photodynamic approach, which is targeted primarily at the lipid components of the viral membrane, and which results in cross-linking of both lipids and proteins, we have chosen the trans-membrane proteins as the specific targets of the photochemically activated species. Cross-linking of envelope proteins has the potential to affect viral infectivity at several levels depending on the manner and extent of cross-linking. It may prevent conformational flexibility of viral proteins essential for viral binding to the target receptor; it may slow the rate of the infection processes (e.g. viral uncoating) enough to allow the normal immune response to prevent infection; or it may provide a physical barrier that completely prevents the delivery of the intact viral genome to the target cell. Somewhat serendipitously, we recently discovered that compounds based on the 3-bromo-4-alkylamino-N-alkyl-1,8-naphthalimide skeleton constitute a new class of hyperchromic photochemically activatable antiviral
agents with $\lambda_{\text{max}} = 420$ nm and $\epsilon_{\text{max}} = 20,000$. These compounds have been singularly successful in neutralizing HIV-1 and in inhibiting HIV-1-induced syncytium formation between HIV-1–infected cells and uninfected cells in vitro, while retaining relatively low toxicity towards uninfected normal cells and extremely low toxicities in the absence of light. Of the compounds tested to date, the monomer naphthalimides represented by 3-bromo-4-(hexyl-amino)-N-hexyl-1,8-naphthalimide (1a) are effective against herpes simplex, type 1 (HSV-1) at concentrations around 250 nM, and the dimeric naphthalimides represented by the bis-amide 2 are effective against the same pathogen at concentrations below 100 nM, both receiving 20 J.cm$^{-2}$ at $\lambda_{\text{max}} \pm 5$ nm. Both classes of dye are also effective in neutralizing the New Jersey and Indiana strains of vesicular stomatitis virus (VSV)$^6$ and bovine herpes virus type 1 (BHV-1)$^7$ at the same light dose.

Results and Discussion

These naphthalimide dyes are extremely hydrophobic and they bind preferentially to lipid membranes, with moderate selectivity for high-cholesterol membranes. The dimeric naphthalimide dye 2 exhibits no acute toxicity to MT-4 cells or to normal human peripheral blood mononuclear cells (PBMC) in vitro in the absence of light, although some light toxicity is observed in the presence of high concentrations of the dye. The requirement for light in the inactivation of HIV-1 by the halonaphthalimide dyes is absolute. In fact, in all viral systems studied to date, including HIV-1, no viral inactivation occurs in the absence of light, and there is a direct correlation between the light dose (i.e. the extent of photochemical bleaching of the dye) and the extent of viral inactivation.

Viral inactivation experiments in vitro were carried out by incubating the virus with the dye solution for 30 minutes at room temperature and then using the treated virus or control to infect cells in culture medium. Cell death and infectivity were assessed by published procedures. Upon irradiation, the dimeric naphthalimide 2 causes viral inactivation in a dose-dependent manner, with a threshold concentration of approximately 0.38 $\mu$M required for neutralization of HIV-1 infectivity towards MT-4 cells following 20 J.cm$^{-2}$ light exposure. Table 1 contains the results of a representative set of experiments; the higher values of optical density of the sample at the time of assay indicate higher levels of cell viability and lower levels of viral infectivity. In a similar fashion, the dye 2 is effective in

<table>
<thead>
<tr>
<th>MT-4 cells cultured</th>
<th>dimeric naphthalimide 2 (M)</th>
<th>Irradiation</th>
<th>Optical density (570 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.08 ± 0.09$^a$</td>
</tr>
<tr>
<td>HIV-1</td>
<td>-</td>
<td>+</td>
<td>0.53 ± 0.04</td>
</tr>
<tr>
<td>&quot;</td>
<td>-</td>
<td>-</td>
<td>0.56 ± 0.06</td>
</tr>
<tr>
<td>HIV-1</td>
<td>2.67</td>
<td>+</td>
<td>1.27 ± 0.10</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>-</td>
<td>0.66 ± 0.05</td>
</tr>
<tr>
<td>HIV-1</td>
<td>1.33</td>
<td>+</td>
<td>1.27 ± 0.14</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>-</td>
<td>0.30 ± 0.07</td>
</tr>
<tr>
<td>HIV-1</td>
<td>0.67</td>
<td>+</td>
<td>1.08 ± 0.07</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>-</td>
<td>0.44 ± 0.06</td>
</tr>
<tr>
<td>HIV-1</td>
<td>0.38</td>
<td>+</td>
<td>1.37 ± 0.15</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>-</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>HIV-1</td>
<td>0.15</td>
<td>+</td>
<td>0.96 ± 0.10</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>-</td>
<td>1.10 ± 0.09</td>
</tr>
</tbody>
</table>

$^a$ Mean ± SD optical density of triplicate samples.
neutralizing the infectivity of a primary HIV-1 isolate towards PBMC, as indicated in Table 2, where the synthesis of HIV-1 coat protein (p24) was monitored as a measure of HIV-1 infectivity. In this case, a higher dye concentration (1.3-2.6 μM) was required for complete neutralization of infectivity. More importantly perhaps, at concentrations of 3.9 μM and higher, the ability of treated HIV-1-infected cells to form syncytia with uninfected CD4+ cells is completely inhibited (Figure 1).

Table 2

Neutralization of HIV-1(JR-CSF) Infectivity of Normal Human PBMC

<table>
<thead>
<tr>
<th>HIV-1(JR-CSF) treated with dimeric naphthalimide 2</th>
<th>HIV-1 p24 antigen (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3⁵</td>
</tr>
<tr>
<td>None (+I)⁶</td>
<td>25±3.2⁷</td>
</tr>
<tr>
<td>&quot; (-I)</td>
<td>95±8.1</td>
</tr>
<tr>
<td>1.3 μM (+I)</td>
<td>-</td>
</tr>
<tr>
<td>&quot; (-I)</td>
<td>25±4.1</td>
</tr>
<tr>
<td>2.6 μM (+I)</td>
<td>-</td>
</tr>
<tr>
<td>&quot; (-I)</td>
<td>18±2.4</td>
</tr>
<tr>
<td>5.2 μM (+I)</td>
<td>-</td>
</tr>
<tr>
<td>&quot; (-I)</td>
<td>16±2.1</td>
</tr>
<tr>
<td>10.5 μM (+I)</td>
<td>2±1.2</td>
</tr>
<tr>
<td>&quot; (-I)</td>
<td>18±10.3</td>
</tr>
</tbody>
</table>

a Days of culture following treatment.
b With irradiation.
c Mean ± SD of triplicate HIV-1 p24 antigen capture ELISA determinations.
d Without irradiation.
e Negative indicates p24 levels <0.007 pg/ml which represents the detection limit of HIV-1 p24 ELISA kit.

Following photochemical inactivation of HIV-1¹⁴ mediated by dye 2, the envelope glycoproteins and intimately associated matrix proteins show altered migration patterns on SDS-PAGE electrophoretic analysis, evidence that the inactivation process involves covalent modification of these proteins. Compared to control, the gels of photo-modified proteins show a strong general increase in background, which we attribute to the oligomerization of individual proteins; some proteins show increased electromigration, which we attribute to intramolecular cross-linking that results in the protein remaining more compact under the SDS-PAGE conditions; some protein bands show altered intensity or disappear entirely, which we attribute to partial or complete intermolecular cross-linking of individual protein molecules. Similar results are obtained with VSV (NJ).⁶

Radioimmunoprecipitation analysis (RIPA) of the photochemically modified HIV-1 proteins (Figure 2) shows that the neutralization of infectivity by the dye appears to be due primarily to its ability to block the binding of the HIV-1 envelope protein gp120 to the CD4 cellular receptors. This is shown by the absence of the band due to gp120 from the irradiated samples precipitated with soluble recombinant CD4 receptor (srCD4) prior to analysis with the L117 anti-CD4 antibody. In this experiment, the band due to gp120 is visible only if it retains its capability to bind to srCD4, which is the agent actually precipitated. Note that in lane 3 (where the sample was not irradiated) the gp120 band is easily visible, indicating that it retains its ability to bind to the CD4 receptor, while in lane 4 (where the sample is irradiated) the band for gp120 is absent, indicating that it has lost its ability to bind to the CD4 receptor.
Figure 1. Effects of dimeric naphthalimide 2 on HIV-1-induced syncytium formation. A: SupT1; B: SupT1 + HIV-1-infected H9 cells; C: SupT1 + HIV-1-infected H9 cells treated with 3.9 μM 2, without irradiation; D: SupT1 + HIV-1-infected H9 cells treated with 3.9 μM 2, with irradiation; E: SupT1 + HIV-1-infected H9 cells treated with 0.93 μM 2, without irradiation; F: SupT1 + HIV-1-infected H9 cells treated with 0.93 μM 2, with irradiation.

However, the loss of binding ability cannot be due to wholesale changes in the protein structure – the precipitation of gp120 by HIV-1 seropositive human serum shows no change in either the density or the migration velocity of the gp120 band, whether the virus is irradiated or not.

There are several other protein effects visible in the gels. The high molecular weight regions of the gel become much darker, indicating the presence of much more high molecular weight material; the viral capsid protein, p24, moves to higher molecular weight and migrates as a much broader band; the band due to the protein designated p55 migrates more rapidly in the irradiated samples, perhaps suggesting that cross-linking of this protein results in its migration as a more compact protein on the gel. In polyclonal antibody-labeled gels, the intensities of the bands due to the trans-membrane protein gp41 and the neighboring matrix protein p17 are reduced by at least an order of magnitude, strongly suggesting that these two proteins are covalently linked by the activated form of the naphthalimide.

The classical, oxygen-dependent photodynamic mechanism of antiviral action has been eliminated by kinetic experiments. In cyclohexane solution the bleaching of 1a best fits a first-order kinetic model, with first-order dependence on the light flux also. Likewise, the bleaching of 1a in isopropyl alcohol solution best fits a first-order kinetic model, as does the bleaching in the presence of an equimolar concentration of tryptophan. Moreover, the best-fit first order rate constants for bleaching of both 1a and tryptophan are identical within experimental error, and their magnitude is unaffected by the absence of oxygen (three freeze-thaw cycles, residual oxygen content ≤ 2 μM). These results are inconsistent with photochemical bleaching as a result of oxidation by singlet oxygen.

The photochemical bleaching of the dye, which appears to be a critical step in the inactivation of the virus, does not occur by simple photodimerization or [2+2] photocycloaddition to oleic acid side-chains of the membrane lipids since products of these processes are not isolated from the reaction mixture after bleaching. Moreover, in phospholipid vesicles, bleaching is partially reversible; in the presence of gramicidin a (a trans-membrane channel-forming peptide),8 bleaching is irreversible and all four tryptophan residues are modified. All of the gramicidin photoproducts absorb light in the visible region of the spectrum. However, the 1H NMR spectra of the modified gramicidins are devoid of vinylic proton resonances, although all other resonances expected in the spectrum are present. These observations are consistent with the presence of an unpaired electron in the aromatic system of the
photoprodut, and we propose that a radical such as 3 may be the agent responsible for protein cross-linking in the presence of good single electron donor residues such as tryptophan.

The results of the kinetic bleaching experiments strongly suggest that the mechanism of bleaching in the presence and absence of single electron donor moieties may be different. Our data indicate that the rate of photo-chemical bleaching of 1a in the presence of indole is approximately an order of magnitude faster than in the absence of indole under the same conditions. We propose that the initial binding of the dye to the membrane occurs with localization of the dye molecule in the region of the double bonds of the unsaturated side-chains of the phospholipids. In the α-helical conformation of trans-membrane proteins, tryptophan residues are frequently found at this location, so that the dye is ideally positioned to form a π complex with the protein. Evidence that these halonaphthalimides form π complexes with electron-rich aromatics is afforded by the uv-visible spectrum of dye 1a in the presence of diphenylamine, which is characterized by an intense charge-transfer band at 285 nm.

The details of the mechanism of protein cross-linking are still under active investigation, and what follows is, of necessity, somewhat preliminary and conjectural in its content. We propose that irradiation at 420 nm of either the dye alone or its π complex with the indole rings of tryptophan side-chains leads ultimately to the relatively stable arylamino free radical 3, possibly via a triplet-state radical pair; this free radical is the key intermediate in the protein cross-linking. We then propose that this radical adds to the indole rings of tryptophan side-chains to give a radical 4 from which the loss of hydrogen bromide should occur readily to give an even more stable radical 5.

A similar mechanism can be written using the side chains of methionine, cystine and tyrosine residues, and it is possible that these residues, also, are important in the cross-linking of viral envelope and matrix proteins. Published literature sequences for the trans-membrane proteins of the viral envelopes of HIV-1 and VSV (NJ) show that all contain at least one tryptophan, tyrosine, methionine or cystine residue in the membrane-associated domain of the protein.

Whatever their other effects, clearly the protein modifications caused by this mechanism must result in viral inactivation. With the dimeric dye 2, cross-linking of proteins could potentially have two major effects. If the covalent cross-links between trans-membrane proteins are dense enough, the delivery of the nucleocapsid to the target cell may be physically prevented. Alternatively, covalent cross-linking of the proteins may retard or prevent certain con-
formational changes in the trans-membrane proteins critical for binding of the virus to the target receptor or for viral uncoating. With the monomeric dye 1a, the latter of these two effects must operate. In the absence of two reactive sites, covalent cross-linking of the proteins is unlikely; thus, inactivation of the virus would be the result of slowing those conformational changes essential for normal binding due to the increased steric bulk of the photochemically modified side-chains of trans-membrane proteins.

In conclusion, the halonaphthalimides offer a new class of photochemically active dyes which show considerable promise for use as oxygen-independent photochemical inactivators of enveloped viruses.

Acknowledgments

We thank Dr. D.J. Hurley and Mr. B.K. Walker of the Microbiology Department at South Dakota State University for permission to quote their results against BHV-1 prior to publication.

The financial support of MicroBioMed Corporation, the South Dakota Governor's Office of Economic Development—CITE, the Office of Naval Research and the National Institutes of Health is gratefully acknowledged.

References


About the Authors

David E. Lewis obtained his Ph.D. in organic chemistry from the University of Adelaide, South Australia, in 1980, and he is currently Professor of Chemistry at South Dakota State University. Address any comments to him.

Ronald E. Utech obtained his Ph.D. in chemistry from Iowa State University in 1986, and he is currently Associate Professor of Chemistry at South Dakota State University.

Millard M. Judy obtained his Ph.D. in physics from the Colorado School of Mines in 1969, and he is currently Scientific Director of the Center for Advanced Laser Applications at Baylor Research Institute.

James L. Matthews obtained his Ph.D. in physiology from the University of Illinois in 1955, and he is currently Director of Photobiology Research at Baylor Research Institute.

Tran C. Chanh obtained his Ph.D. in microbiology from the University of Hawaii in 1980, and he is currently a scientist in the Department of Virology and Immunology at Southwest Foundation for Biomedical Research and a Member of the Center for AIDS Research at SFBR.
Applications of Enhanced Chemiluminescence from Enzyme-Triggerable 1,2-Dioxetanes


In the six-year period since the report of the development of the first stable 1,2-dioxetanes\(^1\)\(^-\)\(^3\) which are chemically and enzymatically triggerable to produce chemiluminescence, these novel compounds have proven to be extremely useful in a variety of applications. The fields of life science research and medical diagnostics, in particular, have made rapid and extensive use of dioxetane chemiluminescence for the qualitative and quantitative detection of biological molecules. Reported examples include enzyme immunoassays for quantitation of drug and hormone levels in the body, DNA profiling in forensic work and paternity testing, DNA sequencing for genome mapping, virus detection and microbe screening. Chemiluminescent detection with enzyme-triggerable dioxetanes offers much greater sensitivity than colorimetric or fluorimetric methods and eliminates the hazards of exposure, limited shelf life and disposal problems of radioactive labelled materials.

Enhanced Chemiluminescent Detection of Alkaline Phosphatase

Lumigen\(^\circledR\) PPD (1a), a phenylphosphate-substituted dioxetane, is an excellent substrate for the enzyme alkaline phosphatase and has proven to be a highly useful substance in biomedical analysis. The utility of this dioxetane derives, in part, from its high stability both in crystalline form and in aqueous solutions, including amine buffers. Of additional significance is the fact that the chemiluminescent decomposition of this dioxetane can be controlled by enzymatic removal of the phosphate protecting group. Reaction of Lumigen PPD with alkaline phosphatase in various buffers at a pH above 9 at 37\(^\circ\)C causes rapid removal of the phosphate moiety with generation of the chemiluminescent aryloxy intermediate (1b). The aryloxy-substituted dioxetane intermediate subsequently decomposes to adamantanolone and the singlet excited state of the anion of methyl 3-hydroxybenzoate. Decay of the excited state results in emission of a visible photon with a quantum yield that is highly dependent on the medium.

Alkaline phosphatase is one of the most widely used labels in enzyme-linked immunoassays and nucleic acid probe assays. The use of enzyme-conjugated reporter molecules and chemiluminescent substrates to provide a detectable signal has allowed the replacement of detection methods based on the use of radiolabelled compounds. The rapid turnover and high affinity of this enzyme for Lumigen PPD (\(k_{\text{cat}} = 4100\) s\(^{-1}\), \(K_{M} = 0.1\)mM), coupled with the ability to detect low levels of light, permit the ultrasensitive detection of alkaline phosphatase.\(^4\)

The utility of Lumigen PPD in aqueous solutions and on solid surfaces can be extended by employing specially formulated reagents containing materials which enhance the quantum yield of chemiluminescence by increasing the light produced in the decomposition reaction of 1b. Further benefits may result from decreasing the background or weak non-specific chemiluminescence caused by slow thermolysis of the dioxetane. Analytical sensitivity is thereby enhanced by increasing the signal-to-background ratio. Enhanced formulations can also amplify the amount of emitted light through the use of energy transfer to an efficient fluorescer.\(^5\)

\[\text{O-O} \quad \text{OCH}_3 \quad \text{OPO}_3\text{Na}_2 \quad \text{alkaline phosphatase} \quad \text{pH 9.6 buffer} \quad \text{O-O} \quad \text{OCH}_3 \quad \text{light}\]

1a (Lumigen\(^\circledR\) PPD)  1b
Enhancement of the chemiluminescence quantum yield is thought to occur as a result of the aryloxide dioxytetan (1b) decomposing in a relatively hydrophobic environment such as is provided by the interior region of a micelle or other organized surfactant assembly. The increase in the amount of emitted light is due to an increase in the fluorescence quantum yield of the emitter and possibly to a higher chemiexcitation quantum yield, i.e. a greater proportion of ester decomposition product is generated in the excited state. Support for this explanation is provided by the observation that the chemiluminescence quantum yield of aryloxide-substituted dioxytanes in pure buffer or other protic solvents is markedly diminished relative to aprotic solvents. Further support comes from a comparison of the rates of decomposition of 1b measured via the decay of light emission. The half life of 1b in pH 9.6 buffer is 3 minutes at 37°C. In the presence of 1 mM CTAB, the half life slows to approximately 13 minutes. Similar slowing of the rate of decomposition of 1b has been observed in solvents of lower polarity than water.

Lumigen has developed several enhanced formulations which enable the ultrasensitive detection of alkaline phosphatase conjugates in a host of different applications and detection formats. The wide array of experiments for which enzyme-triggerable dioxytanes are being used demands that preparations with differing characteristics and advantages be available. As was discussed above, it is generally desirable to have as high a chemiluminescence quantum yield and as low a reagent background as is practical. Having the ability to shift the emission wavelength is often useful and will be discussed below. The kinetics of the reactions leading up to light emission also have a significant influence on the signal produced at a specified time point, since light intensity is a rate measurement. Duration of chemiluminescence can be an important parameter in Southern and Western blotting applications requiring extended measurement times. Specific effects of the test medium, such as the membranes which are extensively used in blotting applications, must be considered in determining the best enhancer system.

The relative advantage of one composition over the other is partly determined by the properties of the detection device being used. Commercial test tube and microplate luminometers vary widely in their spectral response curves; X-ray films and charge-coupled devices used in analyses have radically different wavelength responses. In addition, the maximum and minimum measurable light intensity varies considerably among detection devices so that no single reagent formulation can be optimum for all uses. Ultimately, the choice of reagent and enhancement system should be determined by experiment.

Lumi-Phos® 530 is an enhanced formulation consisting of Lumigen PPD in 0.75 M 2-amino-2-methyl-1-propanol buffer, pH 9.6, containing fluorescent micelles of cetyltrimethylammonium bromide (CTAB) and 5-(N-tetradecanoylamino)fluorescein. Use of this system in the reaction with alkaline phosphatase increases the chemiluminescence efficiency to 0.48%, an increase of a factor of 400 compared to the value in pure buffer. As little as 10⁻²¹ moles of alkaline phosphatase can be detected in solution under these conditions. A companion reagent, Lumi-Phos 480, which lacks the fluorescein derivative, produces chemiluminescence whose spectrum is maximal at 480 nm. Absolute light intensities are somewhat lower in the presence of alkaline phosphatase than those obtained using Lumi-Phos 530. The lower reagent background, however, results in nearly identical analytical sensitivity (S/B).

Numerous applications of the chemiluminescent detection of conjugated alkaline phosphatase using Lumi-Phos have been reported. The sensitivity of a chemiluminescent Western blot analysis of a DNA replication protein was improved ten-fold over a colorimetric method. A Southern blot analysis of DNA on nylon membranes, a Northern blot analysis of RNA, and an enzyme immunoassay for human interleukin IL-6 in serum are typical examples which have recently been developed. Nucleic acid hybridization assays employ a small length of single stranded DNA, which has been chemically attached to an enzyme, to bind and detect the presence of a particular segment of viral or bacterial DNA. Such assays have been developed using Lumi-Phos in a microplate format for the detection of cytomegalovirus, Chlamydia trachomatis, and PCR amplified HIV-1.

Alkaline phosphatase conjugates immobilized on nylon or PVDF membranes detected with Lumi-Phos 530 and X-ray film provides more rapid results and sensitivity equal to or better than radioisotopic methods. The use of Lumi-Phos 530 with nitrocellulose membranes, however, requires significantly longer exposures. The relatively hydrophilic character of the nitrocellulose surface does not provide an environment for efficient generation of chemiluminescence in this system. A new experimental formulation, which we have designated Lumi-Phos EXL, enhances the generation of chemiluminescence on nitrocellulose. This reagent has been applied to the chemiluminescent detection of Western blotted protein and Southern blotted DNA on nitrocellulose.

In a Western blot analysis of human transferrin, dilutions of human transferrin ranging from 5 ng to 20 pg were separated by polyacrylamide gel electrophoresis. After transfer to nitrocellulose membrane, the membrane was
blocked with BSA, incubated with a primary antibody and then with a secondary antibody-alkaline phosphatase conjugate. Lumi-Phos EXL leads to faster and stronger development of signal on nitrocellulose membrane than Lumi-Phos 530. Similar rapid detection of blots on PVDF membrane is also possible.15

In a Southern blot analysis of mouse genomic DNA, the DNA was digested with EcoRI and separated by agarose gel electrophoresis. After transfer to nitrocellulose membrane, DNA was hybridized to a biotinylated probe, blocked with BSA and incubated with avidin-alkaline phosphatase conjugate. The single copy gene corresponding to the homologous gene of v-mos was detected using either Lumi-Phos 530 or Lumi-Phos EXL and exposure to X-ray film for varying lengths of time. Detection of the single copy gene was achieved after a 15 min incubation in Lumi-Phos EXL with a 10 min exposure to X-ray film. Similar signal-to-background ratios with Lumi-Phos 530 required a 3.5 h incubation and a 3 day exposure. In the same analysis on nylon membrane, more modest reductions in exposure time were found relative to those for Lumi-Phos 530. These results demonstrate the enhanced development of signal on nitrocellulose with Lumi-Phos EXL.

The increased light emission as demonstrated through reduced exposure times can be attributed to several factors. When measured by a luminometer, Lumi-Phos EXL produces a ten-fold stronger signal in solution than Lumi-Phos 530 (chemiluminescence from Lumi-Phos EXL can be seen by the unaided eye in a darkened room). The signal-to-background ratio is higher for Lumi-Phos EXL since absolute reagent background is lower. The occurrence of quenching or absorption of luminescence seen when using Lumi-Phos 530 on nitrocellulose membranes is prevented with Lumi-Phos EXL. Further enhancement of signal on certain membranes such as nylon may be due to hydrophobic interactions between the dephosphorylated dioxytane intermediate and the membrane.

Although the use of Lumi-Phos EXL leads to stronger emission and somewhat lower reagent background, a potential drawback of this reagent for detection of alkaline phosphatase in solution is its slower reaction kinetics; the time to achieve one half of the maximum light intensity on reaction of 5 x 10^{-18} mol of alkaline phosphatase at 37°C is 16 minutes using Lumi-Phos 530 and 24 minutes using Lumi-Phos EXL.

A new reagent, Lumi-Phos Plus, which confers the same advantages for detection of alkaline phosphatase conjugates on nitrocellulose membranes as Lumi-Phos EXL, has recently been developed. In contrast to the slower grow-in of chemiluminescence intensity observed with Lumi-Phos EXL, Lumi-Phos Plus exhibits the same reaction kinetics as Lumi-Phos 530. An additional advantage of Lumi-Phos Plus is the higher signal-to-background ratio due to a lower reagent background (Figure 1). Further, the maximum emission wavelength of 470 nm is more readily imaged by the most commonly used X-ray film than the 530 nm light emitted by Lumi-Phos 530 and EXL. As a result, imaging of alkaline phosphatase immobilized on all of the commonly used membranes can be per-
formed with substantially shorter exposures. While the replacement of radioactive methods by chemiluminescent detection with Lumi-Phos 530 allowed detection times to be reduced from days to hours, detection using Lumi-Phos Plus often enables excellent images to be obtained in under a minute. The Western blot analysis of human transferrin, as described above, with a polyvinylidene difluoride membrane produced easily detectable bands down to 20 pg of protein in a 5 second exposure using Lumi-Phos Plus. The time required for detection of a single copy gene in human genomic DNA by Southern blotting onto nitrocellulose was reduced from 16 hours using Lumi-Phos 530 to one hour using Lumi-Phos Plus.

Enhanced Chemiluminescent Detection of β-Galactosidase

Lumi-Gal™ 530 is an enhanced formulation for the chemiluminescent detection of β-galactosidase. This enzyme is widely used as a label in enzyme immunoassays and in gene expression studies to monitor transfection efficiency, both applications requiring great sensitivity and a broad dynamic range. Since the normal pH operating range of the enzyme is 6-8 and phenols are deprotonated at pH > 9, a dioxetane-based reagent similar to Lumi-Phos must balance several needs. Lumi-Gal 530 was designed to achieve enhanced chemiluminescence and maintain enzyme function at a pH well above the enzyme’s preferred range. The reagent contains Lumigen GPD (1c), a β-galactoside-protected dioxetane in an enhanced formulation which permits the enzymatic cleavage step and subsequent dioxetane decomposition step to take place. Reaction occurs at pH 9.6 which, due to the special ability of the reagent to stabilize β-galactosidase, results in chemiluminescence that rises to a maximum level in 30-40 minutes at 37°C and remains constant for an extended period of time. Using this reagent we have demonstrated linear detection of β-galactosidase from E. coli over 5-6 orders of magnitude of concentration.

In summary, the use of enhancers has permitted the development of improved reagents for chemiluminescent detection of alkaline phosphatase and β-galactosidase. The availability of several different reagents, each with particular benefits, allows the user to select the one which offers the best performance in a given application. Further development of chemiluminescent reagents for the ultrasensitive detection of enzymes and enzyme conjugates, including the widely used horseradish peroxidase, are in progress.

Lumigen®, Lumi-Phos®, and Lumi-Gal™ are trademarks of Lumigen, Inc.

References


About the Authors

All of the authors are employed at Lumigen Inc. which was founded by Professor A. Paul Schaap, of Wayne State University, to develop, manufacture and market novel chemiluminescent compounds for use in various clinical, life science research and industrial applications. Dr. Schaap, who holds a Ph.D. in organic chemistry from Harvard, is president of Lumigen and a professor of chemistry at Wayne State University.

Hashem Akhavan-Tafti, vice president of research and development, holds a Ph.D. in chemistry from Wayne State University.

Zahra Arghavani is a research scientist working on the development of new chemiluminescent materials. She holds a Ph.D. in organic chemistry from Wayne State University.

Robert A. Eickholt is a research chemist working on the development of new chemiluminescent materials. He holds a B.S. degree from Wayne State University.

Curtis P. Iscaro is a research chemist working on the development of new chemiluminescent materials. He holds a B.S. degree from Wayne State University.

Renuka Desilva is a research scientist working on the development of new chemiluminescent materials. She holds a Ph.D. in chemistry from Wayne State University.

Richard S. Handley is the director of production and development. He holds a Ph.D. degree in organic chemistry from Wayne State University and previously was employed by the Upjohn Company and BASF Corporation.

S.M. Khaledur Rashid is a research scientist in charge of product manufacturing and quality control. He holds a Ph.D. in organic chemistry from Wayne State University.

Barry Schoenfelsner is a research scientist who specializes in computerization and instrumentation. He holds a Ph.D. degree in inorganic chemistry from Wayne State University.

Katsuaki Sugioka is working in the area of molecular biology research. He received his Ph.D. in Medicine from Hiroshima University in Japan.

Yumiko Sugioka also works in molecular biology. She holds a Ph.D. from Tokyo University and an M.D. degree from Gunma University in Japan.

The production and mailing of The Spectrum is made possible through a generous donation from

The DuPont Company, Wilmington Delaware
Picosecond Transient Raman Studies of Polyene Photoisomerization

Daniel L. Morris, Jr. and Terry L. Gustafson, Department of Chemistry, The Ohio State University, Columbus, OH 43210

Abstract

Photoisomerization reactions involving polyenes are ubiquitous. Picosecond transient Raman spectroscopy is a powerful tool for obtaining direct structural information about photochemical and photobiological isomerization reactions. Among the types of information that can be obtained from transient Raman spectroscopy are the mechanism of isomerization and the nature of the vibronic coupling among states. Some recent, important results concerning polyene photoisomerization obtained using time-resolved Raman spectroscopy in the picosecond time domain are presented.

Introduction

The photoisomerization of conjugated polyenes has received much attention due to the important role they have in many biological processes. For example, the cis-trans isomerization of retinal (Figure 1) is one of the first steps in the visual process, and β-carotene (Figure 1) acts as a light harvesting molecule, transferring energy to chlorophyll in photosynthesis. β-carotene also provides vital protection to photosynthetic systems by quenching singlet oxygen directly or by quenching triplet state chlorophyll from which singlet oxygen is produced. Studies of the electronic state ordering and structure of polyenes have provided a more complete understanding of how they function in biological systems. The symmetry of the ground and first excited electronic states of totally symmetric polyenes (i.e., $C_{2h}$ symmetry) with a conjugation of at least three double bonds is $^1A_g$. It is also known that there is a $^1B_u$ state that lies above the $^2A_g$ excited state. The absolute energy of the $^2A_g$ state and the magnitude of the energy gap between it and the $^1B_u$ state are a focus of many research efforts.

Ultrafast spectroscopy has contributed much to our understanding of polyene photoisomerization. In ultrafast transient absorption studies of β-carotene and related molecules the excited state lifetimes range from 5 to 25 ps. The fluorescence quantum yields are significantly less than the values of the radiative rate constants determined from the $^1B_u \rightarrow ^1A_g$ transition. These data are consistent with the assignment of the lowest excited singlet state in these and other similar polyenes to a state of $A_g$ symmetry. It was also observed that shorter $S_1$ lifetimes result from increasing the unsaturation of polyenes. These data support the suggestion that the $S_1$ decay is controlled by rapid internal conversion.
Transient absorption and emission studies have also yielded information on the vibronic coupling of states in polyenes. High resolution fluorescence spectra show large frequency differences in the C=C stretch in the \( S_0 \) and \( S_1 \) states of short polyenes (≤8 C=C bonds).\(^{11-15}\) In particular, the \( S_0 \) C=C stretching frequency decreases as the polyene length increases while the \( S_1 \) C=C stretching frequency increases slightly until the number of conjugated double bonds equals eight. The \( S_1 \) C=C stretching frequency then gradually decreases. The difference between the \( S_0 \) and \( S_1 \) C=C stretching frequency rapidly increases as the polyene becomes shorter and gradually becomes constant in longer polyenes.\(^{16}\) This frequency difference is attributed to strong vibronic coupling between the \( S_0 \) and \( S_1 \) states via the \( a_g \) C=C stretching mode.\(^{11-14,17-26}\) Transient absorption and emission studies also show that the energy of the \( 1B_{\text{uu}} \) state in polyenes is solvent dependent while the energy of the \( 1A_g \) state is virtually unaffected by solvent changes.\(^{5,27-29}\)

While time-resolved emission and absorption spectroscopies have contributed significantly to our understanding of polyene photoisomerization, they are limited in that they are not capable of providing direct structural information about the molecule. Time-resolved Raman spectroscopy, however, is capable of observing directly the structure of the molecule of interest on timescales as short as picoseconds.\(^{30}\) A schematic diagram of the typical transient Raman experiment is shown in Figure 2. The excitation pulse (\( h\nu_{\text{pump}} \)) populates the excited state (\( S_1 \)).

![Figure 2. Energy level diagram depicting a typical transient Raman experiment. \( S_0 \), electronic ground state; \( S_1 \), first excited electronic state; \( S_n \), a higher lying excited electronic state; \( h\nu_{\text{pump}} \), excitation pulse; \( h\nu_{\text{probe}} \), probe pulse; \( h\nu_{\text{Raman}} \), Raman scatter.](image)

After a variable delay the probe pulse (\( h\nu_{\text{probe}} \)) interrogates the structure of the excited state by resonance Raman scattering (\( h\nu_{\text{Raman}} \)). Note that the resonance enhancement process is associated with the \( S_n \rightarrow S_1 \) electronic transition. Time-resolved Raman spectroscopy benefits from the inherent low fluorescence quantum yields of polyenes. In addition to structural information, one also gets information about ionization, conformation, level of vibrational excitation, vibronic coupling of states, and differences in composition from transient Raman spectroscopy. Both the \( S_1 \) and \( T_1 \) states can be probed by transient Raman spectroscopy, each of which are important in the biological function of polyenes. While the \( S_1 \) state can be generated directly by photoexcitation, the \( T_1 \) state can be formed from intersystem crossing (ISC), triplet energy transfer from a sensitizer, or singlet homo- or hetero-fission.\(^{31}\) Time-resolved Raman spectroscopy is a valuable tool for probing how the conformational and configurational changes in polyenes relate to their specific functions. What follows is a representative review of the work that has been done using ps transient Raman spectroscopy to study polyene photoisomerization.
The α,ω-diphenylpolyenes \([C_6H_5(-CH=CH)_nC_6H_5]\) are the most studied of the model systems for polyene photoisomerization.\(^{32}\) Trans-stilbene (tS) is the simplest \(\alpha,\omega\)-diphenylpolyene (n=1) and is, perhaps, one of the most widely studied molecules using time-resolved spectroscopies.\(^{33-35}\) Trans-stilbene has a ground state of \(A_g\) symmetry. However, tS differs from longer diphenylpolyenes (n>3) in that its lowest excited singlet state is of \(B_u\) symmetry. The well-known photoisomerization reaction coordinate for tS, as first proposed by Saltiel,\(^{36}\) involves the activated twisting of the central double bond on the lowest excited state singlet surface (Figure 3). Following Hochstrasser's initial application,\(^{35}\) the rates for the twisting about the central double bond have been modeled using Kramers' theory\(^{37}\) where the motion of the molecule over the barrier is described in terms of Brownian motion on a one dimensional potential surface. The barrier for photoisomerization in solution for \(S_1\) tS is \(\sim 1200\ \text{cm}^{-1}.\)^\(^{38}\)

Picosecond transient Raman spectra of \(S_1\) tS have been obtained by several groups.\(^{39-48}\) The spectra in solution show 18 discernable Raman bands in the 100 to 1800 cm\(^{-1}\) region, all of which can be assigned to \(\pi\) modes of the \(1\)\(B_u\) state. The position of the olefinic C=C stretch shifts from 1639 cm\(^{-1}\) in the ground state to \(-1565\ \text{cm}^{-1}\) in the excited state, indicating a significant weakening of the double bond character in the excited state. The peak positions and bandwidths of \(S_1\) bands associated with the olefin are also dependent upon the solvent and the delay between the pump and probe.\(^{44-48}\) These results have been interpreted as vibrational relaxation via anharmonic coupling to low frequency vibrations,\(^{44,45,47,48}\) although the exact nature of the mechanism is still unclear.\(^{46}\)

A significant amount of work has been done on 1,4-diphenyl-1,3-butadiene (DPB, n=2), primarily because there is ambiguity regarding the symmetry of its lowest excited singlet state. In one-photon absorption studies in a jet, the \(2^1\)\(A_g\) state appears to be the lowest excited singlet state.\(^{49-51}\) In two photon absorption studies in glasses, liquids, and jets the \(2^1\)\(A_g\) state is the lowest excited singlet state.\(^{51-55}\) However, emission studies show that the emitting state of DPB is not very different from the absorbing state (i.e., the \(1^1\)\(B_u\) state).\(^{56}\) Although a consistent interpretation of these results remains to be found, these observations have been explained by solvent-enhanced inversion of the excited state energy levels\(^{57}\) and by mixing of the \(2^1\)\(A_g\) and \(1^1\)\(B_u\) states.\(^{56,58}\) Time-resolved Raman spectra of \(S_1\) DPB have been obtained by several groups.\(^{59-62}\) Wilbrandt et al. assigned the \(S_1\) state as the \(2^1\)\(A_g\)

---

Figure 3. Potential energy diagram of trans-stilbene photoisomerization.
state. However, Gustafson and co-workers assigned the ps transient Raman spectra to the $1^{1}B_u$ state. The present authors have recently obtained $S_1$ Raman spectra of DPB in several solvents at several probe wavelengths. As representative of the type of data we obtain, in Figure 4 we show the $S_1$ spectra of DPB in decane at delays of -50, 20, 50, and 70 ps. Our preliminary analysis of these data suggest that vibrational bands assignable to both the $2^{1}A_g$ and $1^{1}B_u$ states are present. These data suggest that there is significant mixing of the two states in solution.

![Figure 4. S_1 transient Raman spectra of 1,4-diphenyl-1,3-butadiene in decane at delays of -50, 20, 50, and 70 ps over the region from 1000 to 2000 cm$^{-1}$. Pump, 305 nm; Probe, 670 nm; Repetition rate, 1 MHz; Accumulation time, 20 mins.](image)

There have also been some studies on the next two \(\alpha,\omega\)-diphenyldienes: 1,6-diphenyl-1,3,5-hexatriene (DPH, \(n=3\)) and 1,8-diphenyl-1,3,5,7-octatetraene (DPO, \(n=4\)). Both of these compounds behave very much like retinal and the carotenoids. Their ground states are of \(A_g\) symmetry and the lowest excited singlet state is the \(2^{1}A_g\) state. In the case of DPH, the \(2^{1}A_g\) state lies roughly 1000 cm$^{-1}$ below the one-photon allowed \(1^{1}B_u\) state, and the \(2^{1}A_g\) state of DPO lies approximately 2000 cm$^{-1}$ lower than the \(1^{1}B_u\) state. Thus, DPH and DPO manifest the trend of an increasing energy gap between the \(2^{1}A_g\) and \(1^{1}B_u\) states with increasing polyene length. Kasama and co-workers acquired the resonance CARS and CSRS spectra of the \(S_1\) and \(T_1\) states of DPB, DPH, DPO, and 1,10-diphenyl-1,3,5,7,9-decapentaene (DPD, \(n=5\)). While the highest energy skeletal vibration observed in the \(S_1\) state of DPH is at 1620 cm$^{-1}$, an unusually high energy vibration is observed in the \(S_1\) states of DPO (1755 cm$^{-1}$) and DPD (1775 cm$^{-1}$). Kasama and co-workers assign this high energy band to the olefinic C=C stretch in DPO and DPD. The high energy of this vibration is believed to result from effective vibronic coupling of a symmetric chain mode in the \(2^{1}A_g\) state with the ground state.

### Carotenoids

Owing to the important biological roles of carotenoids, picosecond transient Raman spectroscopy has been used to gain information about their excited electronic states and how these states are related to their essential functions. Time-resolved Raman spectra have been obtained for "simple" \(\beta\)-carotene isomers and for more complicated membrane-bound carotenoids. For the all-\(\text{trans}\), 9-cis, 13-cis, and 15-cis isomers of \(\beta\)-carotene, Flashimoto et al. have shown that the \(S_1\) species generated from each isomer has its own unique configuration. Also, the lowest excited singlet state for all of the isomers was assigned as the \(2^{1}A_g\) state. Transient Raman spectroscopy was also used to study the C=C stretching regions of \(\beta\)-carotene, \(\beta\)-apo-8' carotenal, and ethyl-\(\beta\)-apo-8' carotenolate in both the \(S_0\) and \(S_1\) states. The results show that the solvent does not influence the C=C stretch in the \(S_0\) state, but that it does have an effect on its energy in the \(S_1\) state. This solvent dependence is attributed to \(S_1\)-\(S_2\) coupling associated with a
break-down of the C$_{2h}$ symmetry of the ground state. Transient Raman spectroscopy has been used to establish the lowest electronic excited state for carotenoids bound to the chromatophore membrane *Rhodobacter sphaeroides* 2.4.1. These data show that the $2^1A_g$ state is the lowest excited singlet state of the bound carotenoid, in spite of a shift of the $1^1B_u$ state to lower energy, and that the $S_0-S_1$ vibronic coupling is weakened for the membrane-bound carotenoid.

Time-resolved anti-Stokes Raman spectra also yield valuable information. Specifically, time-resolved anti-Stokes Raman bands reflect the thermal populations of the vibrational motions. The role of excess vibrational energy in chromatophores is not fully established, nor has it been widely considered. However, in view of the fact that these levels would have to be populated by the $2^1A_g$ state, the state through which energy transfer to bacteriochlorophylls takes place, vibrationally excited $1^1A_g$ levels may play an important part in the relaxation mechanisms of *in vitro* and *in vivo* carotenoid systems.68,69 In a time-resolved anti-Stokes Raman study of *in vivo* pigment-protein complexes of *Chromatium vinosum* and *in vitro* samples of the isolated carotenoid spirilloxanthin in benzene, both the *in vivo* and the *in vitro* results demonstrate the formation of vibrationally excited $1^1A_g$ levels within 10 ps of optical population of the $1^1B_u$ state.70 Vibrational relaxation in the ground electronic state is complete within 50 ps in both systems. Another time-resolved anti-Stokes Raman study of carotenoids in pigment-protein complexes of photosynthetic bacteria *in vitro* and spirilloxanthin in benzene and toluene solutions *in vitro* quantitatively characterized the $1^1A_g$ vibrational relaxation as related to population dynamics.71 For the *in vitro* samples, the Raman signal reached a maximum ~10 ps after excitation. The signal decayed with a rate of ~15 ps to intensities that reflect a thermalized $1^1A_g$ population. For the *in vivo* samples, the maximum band intensities are found within 8 ps - the cross-correlation time of the pump and probe lasers.

**Retinal**

The reversible photocycle of bacteriorhodopsin (BR) from the purple membrane of *Halobacterium halobium* is the primary system that has been used to study the conformational changes in retinal in a protein environment.72 The visible chromophore in light-adapted BR is all-trans-retinal (BR-570). The primary function of BR is to pump protons across the membrane. This process involves numerous intermediates with differing conformations, some of which are protonated. Recent ps transient Raman studies of BR have provided considerable structural information about the initial conformational changes in retinal following photoexcitation.73-77

As representative of the type of information obtained from picosecond transient Raman spectroscopy of BR, we will consider the study of the J, K, and KL intermediates obtained by Doig et al. using a two-color pump-probe technique with 3 ps time resolution. They obtained Stokes Raman spectra from 0 ps to 13 ns delays and measured anti-Stokes Raman spectra from 0 to 10 ps. The 0 ps spectrum is believed to arise largely from the J intermediate. This intermediate decays with a time constant of ~3 ps.78-79 The spectrum at this delay is marked by an ethylenic stretching frequency of 1518 cm$^{-1}$ and strong hydrogen out-of-plane (HOOP) modes at 1000 and 956 cm$^{-1}$. The frequency of the ethylenic stretch suggests that the absorption of the J intermediate is red shifted.80,81 These data indicate that isomerization causes a reduction in the electrostatic interaction between the Schiff base and a protein counterion, resulting in greater π-electron delocalization. The strong HOOP modes indicate that the J intermediate contains a highly twisted chromophore. The Raman spectrum of the J intermediate is also marked by a highly congested fingerprint region that the authors conclude is not due to twists about single or double bonds. Rather, a strong anti-Stokes signal at 0 ps indicates the presence of vibrationally hot species; the congestion of the fingerprint region is believed to arise from a thermally excited photoproduct. The transient Raman spectrum at a 3 ps delay is assigned to the K intermediate. The fingerprint region of this intermediate has a sharp band at 1189 cm$^{-1}$ which is characteristic of a 13-cis chromophore. These results indicate that the isomerization process is complete within 3 ps. The K spectrum does not exhibit intensity in the HOOP region as is found in the spectrum of the J intermediate, suggesting that the K species is more planar. These data provide evidence that the protein residues adjacent to the chromophore respond quickly to accommodate the new structure of the polyyne (i.e., allowing it to become more planar). The ethylenic stretch is constant at ~1518 cm$^{-1}$ for ~40 ps. This constancy of the ethylenic stretch does not reflect a blue shifted absorption maximum of K as predicted by time-resolved absorption data. However, this shift could arise from the cooling of vibrationally excited species. The anti-Stokes Raman spectra support this explanation by showing that the chromophore in the photoproduct cools in ~2.5 ps. The authors conclude that J is a vibrationally
hot and conformationally twisted 13-cis photoprod-uct. Upon undergoing the J→K transition, the chromophore ex-
periences vibrational cooling and conformational relaxation. The Raman spectrum assigned to the KL species ex-
hibits an ethylenic stretch at 1521 cm\(^{-1}\) and an intense HOOP region. The intensity in the HOOP region suggests
that the protein-chromophore complex experiences a transition where twists are again introduced in the chromo-
phore. This change occurs at much longer times (between 20 and 100 ps) and must be caused by an isomerization-
induced protein conformational change. The authors conclude that K forms KL in ~70 ps. KL appears to be stable
for at least 13 ns. As can be seen from these data, the ps transient Raman spectra of BR provide extremely subtle de-
tails concerning the structures of the photochemical intermediates.

Conclusions

The examples presented here are intended to be representative of the types of information that can be obtained
from ps transient Raman spectroscopy of polyene systems. The ability to unravel subtle structural differences
among photochemical and photobiological reaction intermediates is a powerful adjunct to more "conventional" ul-
trafast methods, such as transient absorption and time-resolved fluorescence spectroscopies. While considerable
progress has been made in our understanding of polyene photoisomerization, more work remains to be done in or-
der to provide further insight into the relationship between structure and function for these important molecules.

Acknowledgments

We acknowledge Coherent, Inc. for the loan of some of the equipment used in these experiments. We ac-
knowledge the National Science Foundation for support of portions of the instrumentation used in this work under
grant CHE-9108384. We also acknowledge The Ohio State University for partial support of this work through the
Seed Grant Program and the Center for Materials Research.

References

Photobiol. 50, 603 (1989).

About the Authors

Daniel L. Morris, Jr. has a B.S. degree in chemistry from East Tennessee State University and a M.S. degree in chemistry from The Ohio State University. He is currently a research associate in the Department of Chemistry.

Terry L. Gustafson has a Ph.D. in chemistry from Purdue University and is currently an Assistant Professor in the Department of Chemistry at The Ohio State University. Address any correspondence to him.

A Letter to Dr. D.C. Neckers, Executive Director, Center for Photochemical Sciences

The following is an excerpt of a letter written to Dr. D.C. Neckers, Executive Director of the Center for Photochemical Sciences, in response to an editorial written by Dr. Neckers in The Spectrum, Volume 5, Issue 4.

"Your recent editorial in Spectrum has come to my attention. While I agree with you that the Chemical Sciences have changed a great deal since your days as a graduate student, you make statements about other disciplines which are plainly wrong. In particular, you report 'the essential elimination of major research areas-nuclear chemistry and nuclear physics, for example.' This statement could not be further from the truth!

The nuclear physics community is continuing to enjoy a period of growth commensurate with our resources. Yes, nuclear physics has evolved considerably since the 1950s. (Due) to the financial reductions imposed on the scientific community by the national economy, we had to make very hard choices in order to maintain the vitality of the field. We have closed small accelerators and encouraged the best scientists to joint collaborative efforts at the larger facilities. At the same time, we have encouraged 'small' physics without accelerators whenever it represented the highest quality investigations. We are operating two major facilities for intermediate energy physics with hadrons at Indiana and heavy ions at Michigan State University. We are close to dedicating a new electron accelerator, CEBAF (Continuous Electron Beam Accelerator Facility) at Newport News, Va., and are making progress in the construction of RHIC (Relativistic Heavy Ion Collider) at Brookhaven National Laboratory. Progress in Nuclear Theory is on a par with experimental work. Most notable, a new National Institute for Nuclear Theory has recently been created at the University of Washington and is already extremely successful in stimulating new ideas and theories.

I suspect that disparaging statements like the one quoted above are probably due to misinformation. Only by appreciating each other's scientific goals, and by supporting the flourishing of basic research in all fields, will the scientific community be able to continue to make progress in understanding the Universe around us and in harnessing this understanding to improve societal needs.

I therefore hope that all scientific endeavors will grow in the future with continuing development of individual disciplines as well as interdisciplinary studies."

Sincerely yours,

Noemie Koller

Chair, Division of Nuclear Physics, American Physical Society
Present and Future Uses of Photochemotherapy in Medicine

Aaron G. Grand and Francis P. Gasparro, Yale University School of Medicine, New Haven, Connecticut 06510

While perhaps not the "Magic Bullet" of twentieth century medicine, photochemotherapy (PCT) is rapidly becoming accepted as an effective method of treatment for many diseases. Until recently, it has been used almost exclusively in dermatology, since skin is easily irradiated without an invasive procedure. However, recent improvements in endoscopes, laparoscopes, and lasers have greatly increased the number of anatomical areas accessible to irradiation, and the ability to control the cytotoxic, mutagenic, and anti-proliferative effects of photosensitizers makes PCT potentially a good therapeutic agent for many disorders which are currently difficult, if not impossible, to treat.

The most prevalent use of PCT is in dermatology for the treatment of psoriasis, a hyperproliferative disorder of the skin which results in the formation of itchy and scaly patches, which may involve the entire skin surface. Therapy usually consists of psoralen plus UVA light (PUVA). This is done by systemic administration of 8-methoxypsoralen (8-MOP), followed by irradiation of the affected skin areas with UVA light.1 Activated 8-MOP binds to DNA, forming mono- and diadducts. Thus, in affected areas the basal keratinocytes undergo adduct formation, which reduces the hyperproliferation underlying the condition.2 When the extent of the disease is limited, unaffected areas of the skin are not affected by this treatment since they are not exposed to the UVA light and therefore do not undergo adduct formation.

Vitiligo, a skin disorder characterized by patches of depigmentation, is another dermatologic condition often treated with PCT. Histological examination of these patches shows a marked absence of melanocytes, although the pathogenesis of the depigmentation remains unclear. A variety of treatments are used for patients with vitiligo, including PUVA. Why PUVA causes repigmentation in some of these patients is not fully understood, but it remains one of the most effective treatments available.3

Photopheresis, an extracorporeal form of PCT, is an accepted form of treatment for CTCL, a slowly progressing cancer of T-lymphocytes in skin. While the mechanisms underlying photopheresis-induced clearing of CTCL in humans have not been fully elucidated, the immune-augmenting effects of PCT appear to play an important role.4 In photopheresis, 8-MOP is administered systemically to the patient and a quantity of blood is drawn. The blood is then centrifuged to separate the erythrocytes from the leukocytes and plasma, which are exposed to UVA light, resulting in adduct formation. The blood is ultimately returned to the patient.

In this case, the goal of treatment is not to prevent leukocytic proliferation, but to modify the antigenic properties of even a very few circulating cancerous cells. These altered antigens begin to be recognized by the patient’s immune system as "foreign" and cause an immune response. Because the immune system reacts with a polyclonal response, some antigens unique to the cancerous cells may also be recognized by the attacking cells as "foreign." Thus, the attacking cells, stimulated by photopheretically-modified leukocytes, often cross-react with the cancerous cells.5,6,7 This cross-reaction of treated and untreated cells has recently been demonstrated in an animal model.8 In a recent study, patients receiving photopheresis for CTCL had a mean survival 30 months longer than patients in other studies. In addition, complete remission was maintained in several patients for over one year. These findings are in stark contrast to those of previous studies, which demonstrated that conventional therapy does not improve survival of such patients.4

Transplant surgeons may have found a role for PCT, too. Rejection after transplantation surgery is still a common phenomenon, despite the advent of tissue typing and the development of immunosuppressive drugs. Perfect tissue type matches in the MHC complex are virtually impossible, and the use of immunosuppressive drugs such as cyclosporine or cortisone analogs predisposes the transplant recipient to a wide variety of complications, including secondary malignancies and opportunistic bacterial, viral, and fungal infections.9 Often it is these opportunistic infections, and not rejection of the transplanted organ, that ultimately cause the recipient’s death.

Organ rejection is a T-cell mediated response and therefore may be susceptible to treatment by photopheresis in the same manner that CTCL responds to this therapy. As in CTCL, photopheresis selectively attacks or renders
anergic the responsible leukocyte populations. This method of treatment, in addition to reducing the risk of rejection, greatly reduces the need for immunosuppressive drugs, thereby reducing the risk of opportunistic infection. Clinical trials of heart transplant recipients involving photopheresis and endomyocardial biopsy have determined that organ rejection can be stopped with no adverse effects, even in patients undergoing full rejection episodes.10-15

Perhaps the most promising future use of PCT appears to be in a related area: the treatment of cardiovascular disease, the leading cause of death in the United States. Approximately 250,000 coronary artery bypass graft operations and 300,000 percutaneous transluminal coronary angioplasties are performed annually for the treatment of ischemic heart disease. Despite the established role of these modalities, long-term efficacy is limited by restenosis, which occurs in 30-50% of patients,16,17 and which is not preventable by conventional treatment. Evidence from animal, autopsy, and atherectomy studies demonstrates that restenosis is a result of neointimal formation due to migration and proliferation of smooth muscle cells and an elaboration of the extracellular matrix.18-21 A method of treatment which is capable of reducing this neointimal formation would therefore represent a major advance in cardiovascular medicine.

The potential application of PCT in this area is based on the theory that the local inhibition of smooth muscle cell proliferation would reduce the degree of neointimal formation and, therefore, the incidence of restenosis. Many drugs, including heparin, lovastatin, and platelet-derived growth factor antagonists, have been shown to reduce smooth muscle cell proliferation and migration in vitro.22 However, these therapeutic agents have not been clinically effective in preventing neointimal hyperplasia. Hematoporphyrin derivative, while effective in reducing smooth muscle cell proliferation in vitro, results in significant skin photosensitivity for prolonged periods.23 PUVA has also been reported to inhibit smooth muscle cell proliferation.24,25 However, in addition to the potential detriment of prolonged cytostatic effects of PUVA, the penetration of UV light is limited and diaduct formation may be mutagenic.26 The use of PCT with visible light and 8-MOP, an FDA approved drug, may have the potential to safely prevent neointimal hyperplasia: it virtually eliminates the period of photosensitivity caused by hematoporphyrin derivative and greatly reduces the potential mutagenicity of PUVA due to the exclusive formation of 4′5′ monoadducts.

Current technology makes local intravascular drug and light delivery feasible. 8-MOP or another photosensitizer could be administered via an intra-arterial catheter with a dual balloon, resulting in very high local drug concentration.27 Use of such a catheter in both normal and balloon-injured atherosclerotic arteries has demonstrated that transfection of vascular cells with foreign DNA is possible.28 The same catheter should prove to be no less effective in delivering a photosensitizer. Intra-arterial irradiation is also possible with the use of current optical fiber technology. A fiberoptic angioplasty balloon catheter (Spears Laser Balloon Catheter, USCI, Inc., Billerica, MA) has been developed for simultaneous balloon inflation and intra-arterial irradiation. Although designed for high-power infrared laser irradiation, low-power UV or visible laser light could instead be transmitted through the optical fiber to the diffusing tip within the transparent balloon material.

In this scenario, PCT has the potential to greatly reduce or eliminate restenosis after angioplasty or bypass. The ability to exclusively form monoadducts will potentially allow the prevention of cellular proliferation while greatly decreasing the mutagenicity of PCT. It is speculated that by preventing cellular proliferation for several days, PCT allows time for degradation of the cytokines responsible for the stress response. Thus, seven to ten days later, when the cells are once again capable of division, the stimulus to do so will be greatly reduced.

Because of its ability to effectively treat hyperplastic immune states such as CTCL and highly active immune states such as organ rejection, PCT may also be capable of treating autoimmune diseases. These diseases are characterized by the immune system no longer recognizing the host as "self" and therefore attacking it. Systemic sclerosis, systemic lupus erythematosus, and rheumatoid arthritis are examples of some fairly common autoimmune diseases. A preliminary study of the use of photopheresis in systemic sclerosis demonstrated that it has very few side effects and yet is capable of significantly reducing the severity of the disease.29 It is hoped that similar treatment efficacy can be achieved for systemic lupus erythematosus, rheumatoid arthritis, and other autoimmune diseases.

Patients with AIDS suffer from a loss of their CD4 T-cells. This diminished white cell count results in a state of immunosuppression very similar to that sometimes achieved after transplantation. Photopheresis has helped some patients in small, uncontrolled trials, resulting in improvement of symptoms, maintenance of white cell counts, and diminished susceptibility to opportunistic pathogens.30 Although the mechanism underlying these improvements is not understood, PUVA has been shown to result in viral inactivation in platelet concentrates. PCT and adduct formation may therefore be at the heart of a new form of therapy for incurable viral infections.31
Photodynamic therapy (PDT) is similar to PCT but is used specifically for its cytotoxic effects. Most PDT photosensitizers are porphyrin-based, making them uniquely capable of absorbing photons and then transferring that energy to generate highly cytotoxic compounds, such as superoxides and free radicals. While the use of PDT has been limited by the inability to irradiate porphyrin-based photosensitizers through blood, it has nevertheless been used clinically in the treatment of several types of cancers, and may have other uses.32,33

PDT is currently used clinically in the treatment of some cancers of the bladder and skin. It is sometimes also used to remove endobronchial or esophageal obstructions.31 These disorders, unlike psoriasis, will not respond to a temporary reduction in cell proliferation, but require treatment by highly cytotoxic substances. PDT allows for the systemic administration of such substances with only local activation by endoscopic irradiation.

Treatment of neoplasms may someday be augmented by such photosensitizers in two ways. Present treatment for most forms of cancer involves intravenous administration of chemotherapeutic agents which are highly toxic to all cells of the body. Common side effects of chemotherapy include diminished platelet and white cell production, toxic effects on the kidneys and auditory nerves, hair loss, and nausea and vomiting. In addition, chemotherapy is often marked by limited efficacy. However, in a scenario similar to current PDT modalities, drugs which are highly cytotoxic only when activated by light may potentially be administered systemically to the patient. Then light could be delivered focally to the tumor site, resulting in highly specific areas of necrosis. Cancers involving the eye, especially malignant melanoma and retinoblastoma, are uniquely accessible to irradiation, making them particularly good candidates for such therapy. Recent improvements in endoscopes, laparoscopes, and lasers have greatly increased the number of anatomical areas accessible to irradiation, which makes PDT potentially useful in the treatment of many types of cancer virtually anywhere in the body.

Alternatively, the mechanism by which PDT is used to treat CTCL may allow it to be used to treat other neoplasms. That is, its ability to cause changes in antigenicity results in the generation of an immune response to the cancerous cells as well as the modified cells. Such an immune response could eliminate the need for systemic administration of toxic chemotherapeutic drugs.

Summary

It has been known for over two decades that psoralen treatment is based on the formation of cell-modulating DNA adducts.34,35 Activation of psoralen by photons results in the formation of two types of monoadducts and a diadduct, or cross-link.

Psoralen adducts cause chromosomal damage, primarily in the form of single-strand breaks in the DNA, with diadducts additionally preventing strand separation.36 They therefore inhibit DNA replication and transcription and activate DNA repair mechanisms.37-42 Active DNA repair affects the antigenic properties of the cells and effectively stops cell proliferation by preventing DNA replication. Experiments demonstrate that 8-MOP in combination with UVA results in the inhibition of proliferation of bovine aorta smooth muscle cells by 40-60% without significant cytotoxicity.16

Monoadducts can be preferentially formed by irradiation with 419 nm or longer wavelength light.43 This is of therapeutic value, since monoadducts alone are capable of inducing the antiproliferative effect seen in PCT and are less mutagenic than diadducts. In vitro studies have shown that normal or near-normal rates of growth return approximately seven to ten days after treatment with 8-MOP and visible light, which makes monoadduct formation an effective method of temporarily stopping cellular proliferation, while minimizing both cytotoxicity and mutagenicity.44

The three types of psoralen photoadducts can be detected and quantified either by reverse-phase high performance liquid chromatography (HPLC) or by competitive ELISA. HPLC analysis of enzymatically hydrolyzed DNA allows for optical quantitation of adducts when the DNA undergoes 0.3% modification. However, treatment with physiological doses of 8-MOP (100 ng/ml) results in the reduction of the extent of photoadduct formation by two to three orders of magnitude. Therefore, the resulting adducts cannot be quantified, except with the use of [3H] 8-MOP and scintillation analysis of HPLC fractions. Competitive ELISA, utilizing monoclonal antibodies specific for psoralen-modified DNA, has been reported to allow quantitation of adducts resulting from physiologic doses of 8-MOP without the use of radioactive tracers,7,45-47 and will therefore become exceedingly important as a method of measuring adduct formation in vivo.
Novel methods of delivery of both light and photosensitizers have improved the ability of physicians to treat diseases which were untreatable, in anatomical regions which were previously inaccessible, with localization of drug activation to the affected region, and reduction in the frequency of occurrence of side effects. Additionally, the ability to produce different types of photoadducts by using different wavelengths of light will allow the physician to reduce or increase the toxicity of the photosensitizing drug as needed. These advances and the development of new photosensitizers may make PCT a very valuable therapeutic tool in many medical fields.

References

44. Sumpio, B.E.; et al.: unpublished results.

About the Authors

Aaron Grand is a second year medical student at Yale University. As part of his MD program, he is conducting research on the effects of photoactivatable agents on smooth muscle cells.

Frank Gasparro is the Director of the Yale University Photobiology Laboratory. His research is focused on the elucidation of cellular events impacted by photoactivated psoralens.

Center for Photochemical Sciences Will Award First Ph.D. Degrees In Photochemical Sciences

The Center for Photochemical Sciences has reached a new milestone this fall. The first two students in our photochemical sciences Ph.D. program have successfully defended their dissertations. Craig Kelly and Lisa Dennis Kelly will receive their degrees at the December graduation. Craig and Lisa are both taking postdoctoral positions in January. A third student, Yubai Bi, will also receive his degree in December. One other student will receive his degree in May, 1994.

Dr. D.C. Neckers, executive director of the Center, and the initiator of the doctoral program in the photochemical sciences, said, "Pride is not a strong enough word to associate with the awarding of our first degrees in the photochemical sciences. Lisa Dennis Kelly (Advisor Michael A.J. Rodgers) and Craig Kelly (Advisors Elliott Blinn and Michael A.J. Rodgers) are extraordinary scientists well prepared for their respective careers. My compliments to the students and their advisors."

Since its inception in 1989, the program has attracted applications from all over the world. There are currently 32 students enrolled in the program.