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ABSTRACT

Session 1: Paper 1

FLUORESCENT LABELS, PROBES, BEADS & THEIR APPLICATION TO LUMINESCENCE-BASED BIOASSAYS & IMAGING

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Introduction

Fluorescent indicators, probes and labels have widely enlarged our knowledge of molecular events and have paved the way to ultrasensitive bioassays that can reach the single molecule level. Our research focuses on the design of two kinds of probes and labels: The first is on labels and probes for time-resolved measurements as, for example, in delayed fluorescence immunoassay. The other is on long-wavelength labels and probes that absorb in the red (or near-infrared), both approaches with the aim to enable bioassays to be performed in real samples that often display a strong fluorescence background at below 500 nm. Many of these labels can also be used to render nanoparticles fluorescent. Cyanines form a group of dyes that combine relatively longwave absorption with comparatively small molecular size, a feature that is desirable for labels in order not to disturb the system to be probed. In addition, the color of cyanines is fairly predictable from its molecular structure. The chemical structure of cyanine dyes can be represented in general form by structure X(-CH=CH)n-CH=Y, where X and Y typically are nitrogen substituents that are part of a heterocyclic ring. One of the two substituents (here X) has to be present in quaternized (cationic) form. The parameter n in X(-CH=CH)n-CH=Y has the largest effect on the absorption maximum(λmax). The number of n typically varies from 0 to 3. It is known that the λmax values of cyanines increase almost linearly (by 100 nm per unit) with n. While the number for n exerts a massive effect on the absorption maxima, spectral fine-tuning can only be accomplished by variation of substituents X and Y. Symmetrical merocyanines (i.e. those where heterocycle X is of the same type as is Y) have been described rather often, and their absorption maxima have been measured, while those of unsymmetrical dyes are widely unknown. A typical example is provided by the commercially available and widely used label Cy-5 whose structure is shown in Fig. 1a.

Otto Wolfbeis is a full Professor of Analytical and Interface Chemistry with a PhD in Chemistry from Univ. of Graz. He has a rich professional teaching experience in various universities across Europe. He has also undertaken many research assignments. He has to his credit over 430 papers. He is the named (co)inventor of about 45 patents. He has edited numerous books in part chapters and in whole. He is also Editor-in-Chief of Microchimica Acta, of the Springer Series on Methods and Applications of Fluorescence & on Chemical Sensors and Biosensors He is on the journal boards of many leading journals globally. He is the Founder, and Chairman of the Board, of Chromeon GmbH (now Motif Chromeon GmbH). He has won many honours including the Sandoz Prize for Chemistry, Feigl Prize for Microanalysis, Merck Prize for Achievements in Sensor Technology, Friedrich-Emich Medal for Molecular Spectroscopy and many others.

Squarylium-Type Protein Labels

We have developed red and near-infrared fluorescent squaraine dyes (Fig. 1b) and have examined spectra and uses as donors and as acceptors, respectively, in fluorescence resonance energy transfer (FRET) immunoassay based on the use of red and near-infrared lasers. The label proteins show quantum yields of around 10% in the free form and up to 68% when covalently bound to proteins.

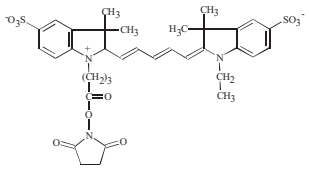


Fig. 1a. Chemical structure of the widely used reactive label Cy-5; λmax(abs.) 647 nm; λmax(em.) 664 nm; ε = 150,000 [L mol^{-1} cm^{-1}]

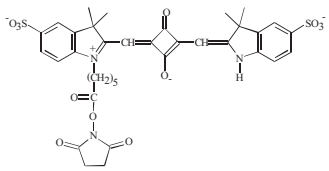
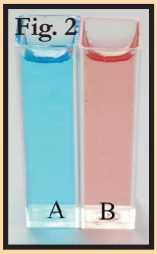


Fig. 1b. Structure of a reactive squarylium NHS ester with λmax(abs.) of 634 nm; λmax(em.) 646 nm; ε= 160,000 [L mol^{-1} cm^{-1}]

Chameleon Labels

A new class of protein stains and label (the so-called Py dyes) were discovered a few years ago. They represent a new class of protein labels that undergo a color change from blue (A) to red (B) on covalent binding to proteins (Fig. 2). While the unreacted stains are almost nonfluorescent (with a quantum yields of <1 %), the protein-conjugated forms are strongly fluorescent. Unlike many other types of labels, the Py dyes do not alter the charge of a protein, and thus does not change its electrophoretic properties. The stains also can be used in quantitative protein assays.



Fluorescent Phosphamidites for Labeling DNA

There is a large interest in labeling oligonucleotides with fluorophores because the resulting fluorescent oligonucleotides are needed for DNA sequencing and DNA hybridization studies. In order to obtain phosphoramidite labels for DNA,[10] a fluorophore containing a hydroxy group is reacted with a phosphine to give the corresponding phosphoramidite.

The dye activated in this way is capable of coupling to the hydroxy group of the (desoxy)ribose of an oligonucleotide. Fig. 3 gives the chemical structures of two new phosphoramidite labels for oligomers. Both can be excited by conventional diode lasers. More importantly, they form a matched pair of labels for FRET studies. The data given in Fig. 2 also document the relatively high molar absorbance and quantum yields of these squaraine dyes. Finally, it becomes obvious that the introduction of a dicyanomethylene group causes a 40-nm bathochromic shift in absorption. One phosphoramidite labels was attached to the 3'-end, the other to the 5'-end so to warrant close spatial proximity of the two labels. Once the duplex is formed, strong FRET is observed.

Diode Laser-Excitable Fluorescent Polymer Beads

Polystyrene beads (of 0.1 - 5 µm diameter) are widely used in immunoassay and in studies on receptor-ligand interactions. In recent years, beads also are being fluorescently dyed for purposes of fluorescent encoding. The color of the fluorescence of the bead, the ratio of two fluorescences of a bead, or the decay time of the fluorophore can serve for identification purposes. We have prepared lipophilic cyanine and squaraine (Fig. 4). They display blue or green color, and we refer to them as the LipoBlue and LipoGreen dyes, respectively. Their chemical structures and spectral maxima are given below. They can be excited by the 635-nm, 670-nm or the 780-nm diode lasers, respectively. A C18 side chain renders them highly lipophilic. They have been used to dye 5-µm polystyrene particles which were first suspended in water/methanol (1:1; v/v), swollen by addition of 2% dichloromethane, and dyed by slow addition of the lipodye in dichloromethane. The resulting beads are of faint blue or green color and display a strong fluorescence that is not quenched by oxygen, proteins, or ions such as halides.

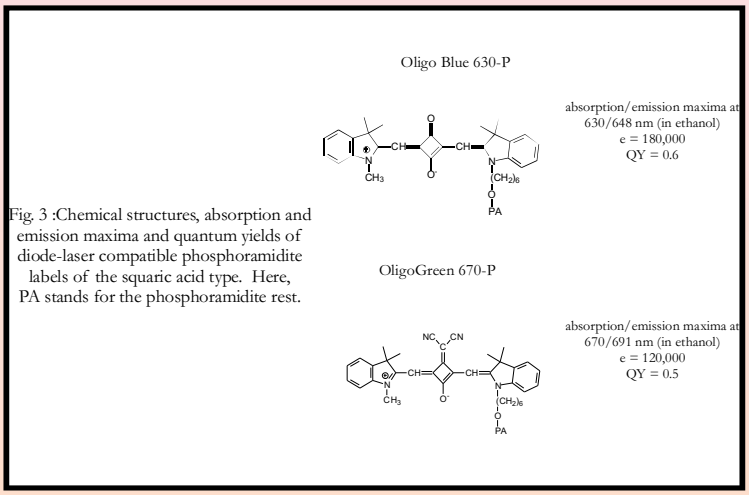


Fig. 3 :Chemical structures, absorption and emission maxima and quantum yields of diode-laser compatible phosphoramidite labels of the squaric acid type. Here, PA stands for the phosphoramidite rest.

Indicator-Loaded Beads for Use in Sensors

We have loaded polymer beads (of 0.1 - 5 µm diameter) with fluorescent indicator dyes for purposes of optical chemical sensing. Polystyrene beads were loaded with oxygen probes such as decacyclene, ruthenium-tris(phenanthroline), or fluorinated platinum porphyrines whose fluorescence is strongly quenched by molecular oxygen. The beads were placed in a polystyrene or hydrogel film of around 2 µm thickness that was deposited on the surface of skin. The fluorescence intensity and decay time of such films strongly depends on oxygen partial pressure. The film was photographed with a camera that divides the complete picture into 526 pixels whose decay times were determined and visualized in pseudo colors. Typical pictures as obtained in tumor research and during photodynamic therapy are shown in Fig. 5. The microbeads have been applied to sensing of chemical parameters. More recently, dual sensing (i.e. sensing two parameters sumultaneously at the same site or sensor spot) such as oxygen, pH, CO_2,but also of temperature, for example in so-called pressure-sensitive paints as they are used in aerospace studies, or for monitoring the growth of bacterial cultures. New fluorescence spectroscopies are needed in many cases.

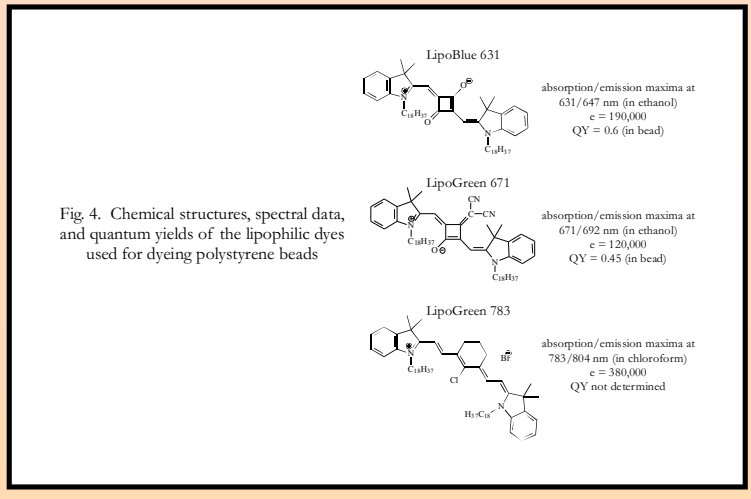


Fig. 4. Chemical structures, spectral data, and quantum yields of the lipophilic dyes used for dyeing polystyrene beads

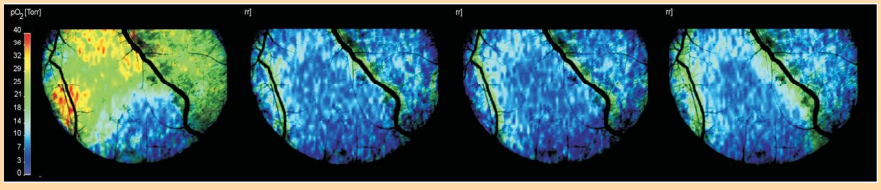


Fig. 5. Imaging the oxygen distribution of the dorsal skinfold chamber with a tumor prior to, and after photodynamic therapy. (a) prior to PDT, (b) after 30 min, (c) after 2 h; (d) after 24 h. Prior to PDT, the tumor region can be clearly differentiated from normal tissue because of its blue color due to the lower oxygen tension. The pO2 is reduced after irradiation in tumor and surrounding tissue.