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Detection and Treatment of Diseases Using Light

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Abstract

Fluorescence light is a natural phenomenon that has been employed recently in medical imaging and therapy. The implication of this technology in clinical studies is largely dependent on the chemical design of organic dyes or photosensitizers. The novel design and application of the probes is the subject of this discussion. In the past decade, significant improvements have yielded innovative dyes for in vivo imaging of numerous diseases in a mouse model while improvements in distribution enable photosensitizers to be used in photodynamic therapy.

Keywords

fluorescence; molecular imaging; near-infrared

I. Introduction

Humans have recognized the emission of light among natural species for thousands of years. However, medical applications of this phenomenon began a little more than a decade ago when scientists initially demonstrated the ability to detect the molecular signatures of cancers in mouse models.

Given the subsequent advancements in chemistry methodologies, several fluorescence probes have been developed for in vivo optical imaging. One of the requirements of this work is the use of near-infrared (NIR) dyes. Imaging in the NIR window offers several advantages compared to blue-shift dyes, including deep tissue penetration and an enhanced signal-to-noise ratio. The two major types of fluorescence probes that dominate this field at present are targeted imaging probes and enzyme-activated probes. Targeted imaging probes involve the labeling of specific ligands to the dyes, while enzyme-activated probes, sometimes called “smart” activated probes, involve the use of fluorescence resonance energy transfer (FRET) to sense the activities of proteases associated with diseases. Since activation is specific to the molecular expression level of the measured biological pathway, this technique is more quantitative and real time in nature.

In addition to imaging, the fluorescence technique can be used to sensitize intrinsic molecules of the tumor microenvironment to generate radicals intended to kill cancers. This technique is called photodynamic therapy and thus far, it has proven remarkably effective for the treatment of surface tumors in clinical studies.

In this paper, we will discuss the development of probes for the imaging and treatment of cancers.

II. Probe development

A. Fundamental of Light

When a small organic chromophore receives light, its electrons will excite from the ground state to the higher energy level excited state. Immediately, the electrons will relax and return to the ground state via several mechanisms depending upon the structure of the molecule. In general, the cascade of electrons to the S_0 state follows a radiation or radiationless mechanism. In the latter, the delivery of electrons from the S_1 energy level to S_0 via internal conversion or intersystem crossing generates heat. One of the applications of this mechanism can be found in photochemistry where the delivered excited molecule to the ground state associated with new bond formation due to the change of the molecule's electronic configuration. In the radiation mechanism, the direct return of the excited electrons from the S_1 to the S_0 energy state emits fluorescence light. In some dyes, the excited electrons cascade to the triplet state and emit light from T_1 to S_0 generate phosphorescence. There is a fundamental difference between fluorescence and phosphorescence. The former emits light over the course of nanoseconds while the latter can last several minutes.

The excited electrons in the triplet state from some dyes, for instance porphyrin, can sensitize the triplet ground state of oxygen into excited state singlet oxygen, which thus becomes toxic for cells. The reactive oxygen species (ROS) have been utilized for the eradication of tumor cells, a process called photodynamic therapy (PDT).

B. Fluorescence Dyes

The chemistry development of colorants has been studied exhaustively in the past century. As a general rule, any extended conjugation compound can absorb light in the visible region and produce color. This is explained by the molecular orbital theory, in which the electrons in the highest occupied molecular orbital (HOMO) (2×10^{-7} m) undergo a transition to the lowest unoccupied molecular orbital (LUMO) (4×10^{-7}). The longer the conjugation system, the greater the shift to the red region, as a consequence the energy difference between HOMO and LUMO decreases. Fluorescence dyes used for molecular imaging not only contain large π -conjugated systems, but other requirements associated with the intrinsic characteristics of optical imaging and tissue penetration are also taken into account. Although none of the dyes can meet all of the requirements, the ideal colorant would include in the design (i) NIR absorption; (ii) light emission; (iii) water solubility; (iv) chemical stability; (v) and last but not least, the availability of a function group for bioconjugation. Currently, cyanine and rhodamine are the most popular family of fluorescence dyes used in molecular imaging.

Cyanine dyes—Cyanine dyes are comprised of a large family of colorants that have been used in textiles, optical media, the Xerox industry, guest-host liquid crystal displays, and recently in molecular imaging and other medical avenues. The chemical structure of cyanine dyes includes either one or two heterocyclic aromatic ring systems joined by an unsaturated chain of carbon atoms called the polymethine bridge (Fig. 1). The elements of oxygen or nitrogen on those rings act as an electron sink where absorbance of the dye relies on the electrons' propagation forward and backward along the methine chain. What makes cyanine dyes stand out uniquely compared to other dyes is the flexible chemistry that enables modifications through many possible positions on the backbones for numerous applications such as, (i) the absorbance wavelengths can be tuned as precisely as possible; (ii) solubility; (iii) stability; and (iv) functionalization.

Tuning near-infrared (NIR) cyanine dyes can be achieved through modulation of the methine carbons or the electronic effect in the end groups. For every additional methine carbon, the wavelength will enhance 100 nm versus 20 nm for an additional phenyl ring. This approach has particular implication when designing dyes for molecular imaging; while the dyes have extended wavelengths, the design retains compacity and hydrophilicity, which represents another advantage of methine group modification.

Another approach to designing a stable dye with NIR capability, although not particularly popular but seemingly feasible is the compromise of long polymethine carbons for shorter ones while optimizing the electronic effect of the aromatic rings. Possible rings are benz[c,d]indolium, telluropyrilium, and cyclohepta pyrrolium salts. This strategy is exemplified by comparing the absorbance bands between indole-based ring and its counterpart, benz[c,d]indole-based ring. Both of the dyes have the tri-methine bridge but the latter excels 180 nm to the red shift compared to the other. This suggests that designing unique heterocyclic end groups is critical in the quest to make NIR dyes.

Overall, cyanine dyes have good physical properties; they have a unique, mirror image between absorbance and emission profiles, and the dyes have an acceptable quantum yield for imaging applications. The Stokes shifts of cyanine dyes are narrow, with most in the range of 30 nm. However, this is not always the case. Recently, chemists have been able to develop cyanine dyes with large Stokes shifts, similar to that of quantum dots. The underlying mechanism of this design focuses on perturbation of the electronic propagation along the polymethine bridge. The intramolecular charge transfer mechanism is attributed to a change of geometry in the emitting and absorbing profiles.

Rhodamine dyes—Rhodamine and oxazine dyes are considered as honeycomb dyes, since they have architectural clusters of hexagonal frameworks formed by the conjugated π -system. Rhodamine dyes are perhaps the most popular in terms of their biological applications in view of their stability. For instance, they are comprised of a family of dyes used in microscopy, histology, and imaging such as Alexa dyes, Fluorescein and Texas Red. The simplest synthesis of rhodamine dye is the condensation of m-diethylaminophenol with formaldehyde, followed by oxidation with ferric chloride in hydrochloric acid. In order to create the dye with a longer wavelength, a few choices are available: (i) enhancement of the ring size or (ii) electronic manipulation on the backbone of the molecule. In the first case, the condensation reaction between large ring structures of m-aminophenol with phthalic anhydride can provide the dye product with emission near the NIR range (Fig. 2). In the second case, NIR rhodamine dyes are achieved by replacing the carboxylphenyl moiety with an electron-withdrawing group at the central carbon. For example, the addition of a CF₂ moiety in place of an aromatic ring resulted in an apparent bathochromic shift.

One of the superior characteristics of rhodamine dyes is their high quantum yield. No other family of dyes can provide such high quantum yield. In general, the quantum yield of rhodamine dyes can reach from 50 to 90%. This may partially be attributed to the stability of the dyes.

Rhodamine dyes that have hydroxyl groups at the end rings are very sensitive to physiological pH change and are used as intracellular pH indicators. For example Vita Blue 9, an amphiphilic dye designed for cell membrane permeable. The deprotonation of the dye in basic condition switches the excitation/emission from 524/609 nm to 570/665 nm.

Oxazine dyes—By replacing the central carbon of a rhodamine with a nitrogen atom, an oxazine dye forms. Because of this replacement, the molecule exhibits longer wavelength (1). The central nitrogen atom serves as an electron sink for the π electrons of the

quarternary amine in the indole ring. This design contributes to a wavelength shift of about 80–100 nm toward the NIR region. There are a number of oxazine dyes emit light in the NIR region (2). Furthermore, several lines of research demonstrated that oxazine dyes are photo-chemically more stable than rhodamine dyes (3).

FRET—Fluorescence resonance energy transfer (FRET) has been used widely in biophysics over the past several years since its discovery four decades ago by Stryer and Haugland. Since the underlying mechanism involves energy transfer from the donor fluorescence molecules to the acceptor dyes when they are in proximity to each other, the dynamic characteristics of this interaction are considered to be the molecular ruler used to determine the spatial distance between two entities. The initial use of FRET between fluorescence proteins revealed crucial information regarding the intracellular interaction, unfolding protein function, and dynamic biochemical signals in living cells.

Another recent scientific employment of FRET focuses on the *in vivo* detection of protease activity. The advent of optical devices, especially the CCD camera and computer-based imaging technology, has provided a straightforward method to detect the dequenching photons from the FRET activation. One typical design of a FRET probe for imaging application can be found in the recent development of fluorescence-activated molecular beacon to detect MMP7 (matrix metalloproteinase). In this construct, the specific peptide backbone for MMP7 was modified to accommodate a FRET pair on the peptide ends. This can be achieved through an orthogonal chemical maneuver in which the acceptor dye was conjugated to the terminal aminated peptidyl resin. While the donor was conjugated in solution phase. During FRET, the transfer of electronic excitation energy from a donor chromophore to an acceptor dye located in a close distance via a through-space dipole-dipole coupling mechanism between the donor-acceptor pair. Treatment of this probe in the presence of MMP-7 afforded a specific enzyme-activation fluorescence that was seven fold higher than the control experiment. *In vivo* imaging of MMP7-associated cancer in a xenograft mouse model confirms the specificity and sensitivity of this design (Fig. 3).

Using a similar strategy, the activity of BACE (β -site of APP-cleaving enzyme), which is an aspartic protease that cleaves the amyloid precursor protein (APP), the β -secretase site was detected at nanogram scale. The activated fluorescence signal was detected conveniently using an optical imaging instrument (Fig. 4).

The selection of a pair of dyes for FRET probes would be more effective if the acceptor dye could act as a dark hole quencher. In this capacity, the dye's exclusive role is to absorb the emission photons from the donor, but would not emit fluorescence light. For example, a few years ago, we developed such quenchers using an azulene backbone. As a matter of fact, it has been known for a while that azulene derivatives have anomalous fluorescence due to the large S1–S2 gap, which thus prevents excited photons from equilibrating to the S1 energy level. However, the short absorbance wavelength of individual azulene hampers its role in imaging application. To overcome this, we tuned the absorbance of dyes from visible to NIR by extending the conjugated π system utilizing the polymethine carbons. Another interesting aspect of this work is that the dye can be tuned to exert bathochromic shift using the molecular orbital perturbation theory, with particular applicability to the π -electrons of the aromatic end rings (Fig. 5).

Photodynamic therapy—Three components are involved in PDT (i) photosensitizers; (ii) light; and (iii) reactive oxygen species. Organic dyes with unique excitation photons that cascade in the triplet state have profound implications for therapy to eradicate tumor cells because of their specificity and sensitivity. Similar to the imaging approach, one of the requirements in PDT is that light must reach all of the cancer tissue for successful therapy.

This is a challenging, since light may either scatter or be absorbed by intrinsic tissue chromophores. Therefore, the photosensitizers used in PDT must have a long absorbance wavelength that permits deep tissue penetration, with minimum scattering and maximum photosensitizer activation, and the greatest tissue destruction (4). The super family of porphyrin dyes were one of the earliest photosensitizers used in PDT. Although there is no ideal photosensitizers available currently, porphyrin compounds seems to be the best candidates given the ease in modification on the compounds for in vivo study. The dyes also have acceptable triplet excited state long-live enough to produce high quantum yields of singlet oxygen. To extend the absorbance of porphyrin, several works have demonstrated that hydrogenation of one of the pyrrole rings of an porphyrin, which generates a reduced form known as chlorins, have a highly absorbent band in the red region of the spectrum, specifically in the 630–715 nm range. These sensitizers populate in the triplet state with excellent lifetimes of greater than 100 μ s, which are efficiently quenched by ground state oxygen to produce singlet oxygen with a quantum yield of approximately 0.6 (5).

One of the challenges in using porphyrin-based photosensitizers in clinical study is their hydrophobicity. Therefore, the design of photosensitizers for in vivo study would include a technique for distribution either by carriers using liposomes or through the incorporation of intrinsic hydrophilic moiety, such as sulfonate groups. Sometimes, a balance control to keep the compounds to be amphiphilic is the key to optimizing cancer treatment (6). Other techniques that are available to improve tissue availability include metal chelator or cationization to increase cellular uptake through the electrostatic interaction.

Currently, PDT has become an FDA-approved procedure for the treatment a number of cancers. In clinical work, the photosensitizer (Photofrin®) is injected into the bloodstream of the patient. The compounds will traverse to the whole body, but will eventually distribute and reside in the tumor longer than in other tissues. Light will be used to illuminate the photosensitizer a day post injection in order to generate ROS for killing tumor cells. What makes PDT more appealing than other techniques for treatment of tumor is that the production of ROS not only damages tumor cells but also their surrounding network. Tumor growth is associated with angiogenesis, ROS generated in the tumor mass damages the blood vessels, will help to kill tumor cells indirectly due to their lack of nutrients.

Since light absorption is limited in tissue penetration, PDT is suitable for the treatment of surface tumors like melanoma, or on the lining of internal organs such as the lungs or esophagus cancer. The technique is still limited to large tumors.

III. Conclusion

It is increasingly recognized that the development of molecular probes for imaging and PDT plays an important role in modern medicine and research. Besides enhancing our capability to detect the diseases, these probes can be used to monitor and assess therapy in preclinical animal models. There is still enormous opportunity related to research into optical imaging and PDT. Successful implementation of the design of physical and biologically advanced probes, combined with the detection capability, could produce an imaging modality that is more effective and safer than the other modalities currently available.

Acknowledgments

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Fig. 1.
A general chemical structure of cyanine dyes

Fig. 2.
Tuning a rhodamine dye to NIR window

Fig. 3.
(A) Imaging MMP7-associated tumor using a FRET probe; (B) control mice

Fig. 4. Activation of a peptide-based NIR FRET probe with BACE. (A) A fixed concentration of probe was treated with increasing concentration of BACE. (B) Optical imaging of the activation

Fig. 5.
Tuning the absorbance wavelength of azulene-based quenchers