Clinical and Experimental Health Sciences

Review / *Derleme*

Near-Infrared Dyes and Their Use in Medical Science

Yakın Kızıl Ötesi (Near-IR) Boyalar ve Bu Boyaların Tıp Alanında Kullanımları

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Abstract

Targeted imaging (diagnosis) and therapy using near-infrared (NIR) dyes can be accomplished with the help of the data obtained from fluorescence emission of the fluorophores and play an important role particularly in deep tissue imaging. The area NIR dyes absorb and emit light is defined as NIR spectroscopy (NIRS, 650–850 nm). Although NIR dyes are widely used for imagining purposes, they also find application in photodynamic therapy. In preclinical studies, phthalocyanine (Pc), chlorine, porphyrin, bacteriochlorin, cyanine, Alexa-fluor, and various BODIPY dye series are used as NIR fluorescent dyes/agents. When compared to other dyes, one of the most promising NIR dye is Pc because of their photophysical and chemical properties particularly for the imaging applications. Although NIR dyes have several advantages, their toxicity limits their usage in clinics. Indocyanine green, having negligible side effects, is the only FDA approved NIR dye used in clinics. It is used for controlling of cardiac function, liver output, and retinal angiography. In conclusion, the development of new generation NIR dyes with improved chemical, photophysical, and photochemical properties that are more appropriate for the aforementioned applications is inevitable. Nevertheless, the NIR dyes that have been developed and will be developed should be combined with the nanoparticular systems and/or targeting moieties to make them more advantageous for NIRS and therapy.
Keywords: Near-infrared dyes, photodynamic therapy, phthalocyanine, theranostic, targeted imaging, fluorescent probes

Öz

Near-IR (NIR) (yakın kızıl ötesi) floresan boyalarla hedefe yönelik görüntüleme (tanı) ve tedavi imkânı, görüntülerin renk yansıması ve floresan emisyonundan alınan datalar yardımıyla gerçekleştirilir ve özellikle derin yüzeyde bulunan dokuların görüntülenmesinde önemli rol oynar. Bu moleküllerin soğurma ve floresans yaptıkları bölge NIR spektroskopisinin (NIRS) moleküler görüntülemelerdeki seçici alanı olarak tanımlanır (650-850 nm). NIR floresan boyalar genel olarak NIR görüntüleme içeren çalışmalarda kullanılmasına rağmen günümüzde fotodinamik tedavi de kendine yer bulmaktadırlar. Klinik öncesi araştırmalarda, ftalosiyanin, klorin, porfirin, bakterioklorin, siyanin, alexfluore ve çeşitli bodipy serileri vb. NIR floresan boyalar/ajanlar kullanılmaktadır. Bu boyalardan özellikle görüntüleme çalışmalarında en öne çıkanı, diğer boyalara kıyasla ileri fotofiziksel ve kimyasal özelliklere sahip ftalosiyaninlerdir. NIR boyaların kullanılmasının mevcut ve potansiyel avantajlarının yanında bu boyaların toksisite sorunu boyaların klinikte kullanılmasını kısıtlamaktadır. Klinikte kullanılan FDA onaylı tek boya indosiyanin yeşilidir ve ihmal edilebilir yan etkileriyle, kardiyak fonksiyonların kontrolü, karaciğer çıktıları ve retinal anjiyografi gibi klinik alanlarda kullanılmaktadır. Sonuç olarak, birçok önemli sorunlar taşımakla birlikte günümüzde hala güvenli kullanıma uygun olmayan NIR boyaların yakın gelecekte bahsi geçen uygulamalarda uygun olarak kullanılabilecek kimyasal, fotokimyasal ve fotofiziksel özellikleri geliştirilmiş şekilde üretilmesi ihtiyacı kaçınılmazdır. Bununla beraber geliştirilmiş ve/veya geliştirilecek olan NIR floroforların nanopartiküler sistemlerle birleştirilerek ve/veya ajan moleküller ile hedef belirlenerek NIRS ve terapi yönünden daha avantajlı hale getirilmesi gereklidir.
Anahtar Kelimeler: Yakın kızıl ötesi boyalar, fotodinamik terapi, ftalosiyanin, teranostik, hedefe yönelik görüntüleme, floresan sistemler

INTRODUCTION

Near-IR (NIR) Dyes

Fluorescence-based detection provides support to a number of biological assays including but not limited to immunoassays, flow cytometer, DNA sequencing, various proteomics assays, and several clinical chemistry applications. Because of recent advances in the field, the efficiency of these assays has to be amplified. In recent years, when minimal background fluorescence and low scattering in the 650–850 nm region of the electromagnetic spectrum (Figure 1) were coupled with photophysically and chemically improved fluorophores, near-infrared (NIR) dye-based fluorescence imaging gained importance (1, 2). Although the primary purpose of the NIR dyes is aiding imaging, some of the NIR dyes are also used as therapeutic agents for photodynamic therapy (PDT) of cancer and other diseases.

NIR dyes show variety based on their solubility, molar absorptivity, photostability, and fluorescence efficiency. Phthalocyanines (Pcs), cyanines, BODIPY dye series porphyrins, squarins, benzo [c] heterocyclic, and xanthine derivatives are the most commonly used NIR dyes (3-5). Among these dyes, cyanine dyes receive attention because of their high molar absorptivity, strong fluorescence emission, and good photostability. However, their excitation and emission spectra interference as a result of intrinsic small stokes shift is an obstacle for their use as NIR imaging agents (6). Porphyrins are tetrapyrolic compounds. Depending on the substituent pattern on the macrocycle, their chemical

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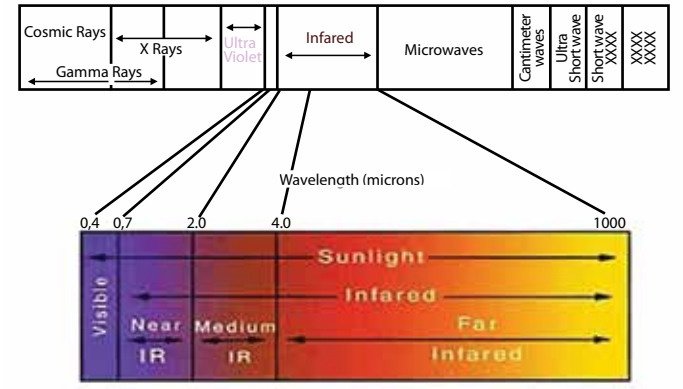

Figure 1. Electromagnetic Spectrum

and photochemical properties can be easily tuned. Instead of being used as NIR imaging dyes, porphyrins are predominantly used for PDT applications because of their ability to form triplet state complexes via intersystem crossing and to generate reactive oxygen species. A derivative of porphyrin, chlorin, is a two electron reduced from of porphyrin and is widely used as a photosensitizer (PS) for PDT applications, which will be further elaborated in the following sections of this review.

Compared to porphyrin derivatives and cyanines, BODIPY series have lower molecular weight (Figure 2) and last within the cells for a longer period of time, which provides a better time scale for imaging. BODIPY dyes have many derivatives having absorption and emission profile ranging from 500 nm to 660 nm in the electromagnetic spectrum (Figure 3). However, typically they have a relatively shorter wavelength emission maxima and smaller extinction coefficients compared to the other NIR-active fluorophores (e.g., Pcs) (7, 8).

Pcs, known systematically as tetraaza tetra benz porphyrins, are a member of porphyrinoid derivative aromatic compounds (Figure 4). Pcs are planar macrocycles with 18 p electrons. In the Pc structure, aza bridges connect four isoindole units linked together through their 1, 3 positions (Figure 4). Pcs have a number of characteristic properties such as high thermal stability, unique photophysical properties, intense color, non-toxicity, and high phototoxicity upon irradiation with light, contributing to their effectiveness in different research areas. Their easily tunable photochemical and photophysical properties such as narrow absorbance and emission band and resistance to photobleaching, by changing the substitution pattern around the Pc aromatic core and/or the central metal atom, makes these compounds highly appropriate for applications such as PCR, single gene mutation detection, and resonance energy transfer-based assays (9).

CLINICAL AND RESEARH CONSEQUENCES

For most of the applications, it is a "must" to have Pcs with high water solubility along with a functional group that can be used for bioconjugation. However, difficulties in their synthesis limit their usage; therefore, a new synthetic method has been developed by Erdem et al. (1, 2) that has been published in two different articles in 2008 and 2009 in the Journal of Organic Chemistry Article (1, 2). In these studies, using a new solid phase synthesis method, Erdem et al. (2) were able to synthesize monoamine and mono-hydroxyl functionalized highly water-soluble Pcs to be used as NIR imaging agents. Further expansion of the study included the conjugation of monoamine

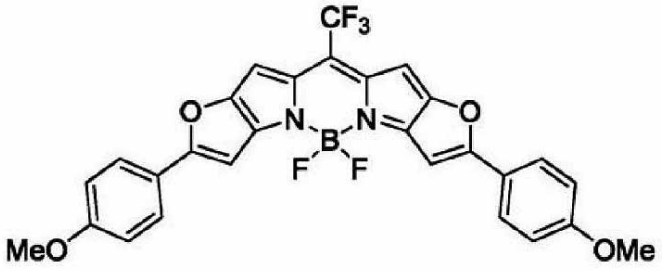

Figure 2. General structure of BODIPY dyes (6)

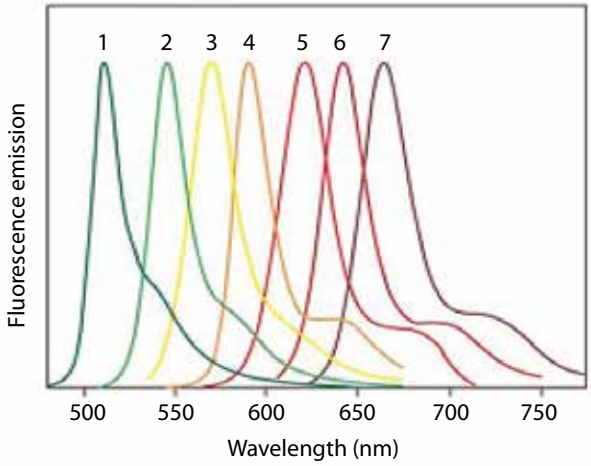

Figure 3. Normalized fluorescence emission spectra of BODIPY series

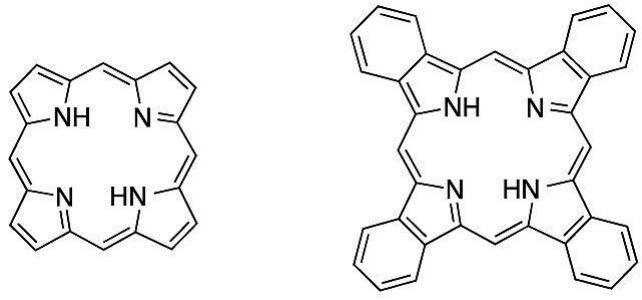

Figure 4. General structure of porphyrin and chlorin

functionalized Pcs to various length oligonucleotides. The authors successfully showed that the synthesis and isolation of the Pc–oligonucleotide conjugate takes just few hours with the help of a three-step specific bioconjugation method.

For many bioanalytical applications, Pcs are used as fluorophores to tag biomolecules because of their structural properties. They are specifically useful fluorescence on-off systems offering high target to background ratio due to Pcs high extinction coefficients and strong and narrow emission profiles. In the light of this information, Nesterova et al. (9) have published another article in the Journal of American Chemical Society in 2009 as a follow up study (9). In this study, authors, for the first time, have designed, prepared, and evaluated the use of double-labeled dimerization-based molecular beacons

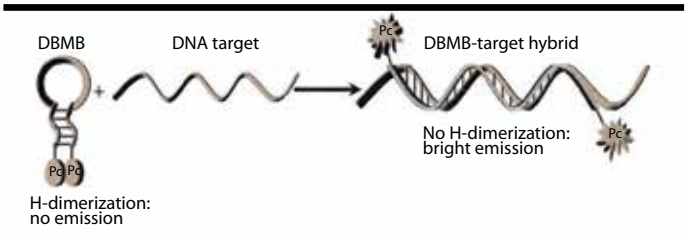

Figure 5. Dimerization-based molecular beacons assay using Pc dyes (9)

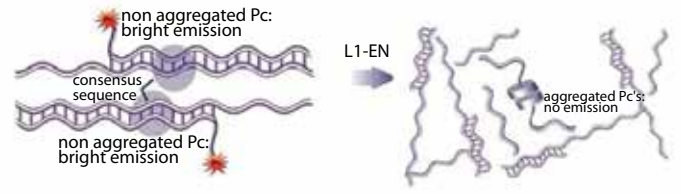

Figure 6. Schematic of DNase action on substrate and emission (10)

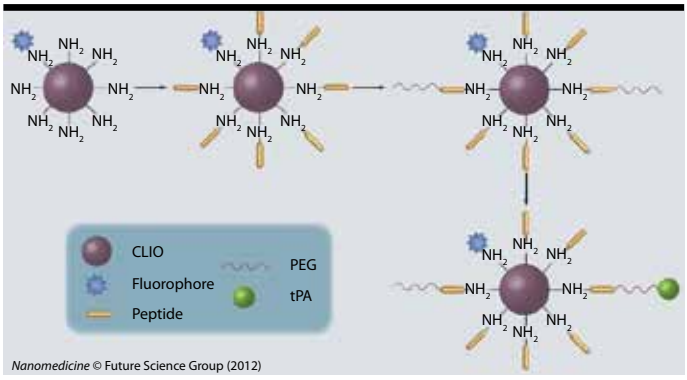

Figure 7. Step-wise synthesis of thrombolytic nano particles synthesis (11)

using Pcs as markers for single nucleotide base pair detection to be used in vivo cell imaging to trace cancer cells. For this purpose, the authors have designed "off/on probe" in a way that two identical Pc molecules in the molecular beacon's closed state form a non-fluorescent H-dimer (off state). However, in the presence of a complementary DNA, the molecular beacon's loop hybridizes to its target forcing the molecular beacons to open, disrupting the Pc dimer and restoring fluorescence emission (on state) (Figure 5) (9). On the other hand, in the presence of a single base-pair mismatch, the loop does not open due to mismatch between the loop and the complimentary DNA, the probe stays in the off state and consequently fluorescence read-out does not get recorded.

A different perspective of Pc aggregation-based system (on/off probe) has been described in another study published in the Analyst in 2010. Nesterova et al. (10) developed a Pc aggregation-based NIR fluorescence quenching system employing only one type of a fluorophore. With the aim of discovering inhibitors for long interspersed Element 1 endonuclease, the authors successfully demonstrated that the probe could effectively distinguish differences in enzyme activity via H-aggregation of single Pc-labeled oligonucleotide. The probe designed in a way that Pc bearing oligonucleotide is used as a fluorescence quenching system. In the generated probe-based system, in the absence of the enzyme, two fluorophores are located far away from each other (on the either end of the double stranded oligonucleotide) so that the fluorescence read out is recorded (on state). As the enzyme is introduced in the system, it cleaves the double stranded oligonucleotide. Consequently, fluorophores (Pc) get close to each other in the solution resulting in quenched fluorescence (off state) (Figure 6). The advantage of using only one type of a fluorophore is reduced cost and time for substrate preparation. The signal read-out format overcomes some disadvantages of the "off/on system." In the signal read-out format, amplification of the signal is monitored against a low background (10). It is concluded that such a system would be particularly beneficial in high throughput screening applications, in which enzyme activity is closely monitored.

Besides on/off or off/on systems, NIR imaging can be coupled with nanoparticles to increase detection efficacy by measuring the number of fluorophores in the tissue of interest. Nanoparticles have many advantages such as tunable pharmacokinetics, increased number of fluorophores per area, and the ability to carry more than one type of a molecule (therapeutic, diagnostic, and biomarker targeted). All these features make nanoparticles an attractive choice as drug carriers for theranostic studies in recent years (in vivo and in vitro). McCarthy et al. (11) study of thrombus targeted fibrinolytic nanoparticles is one of the best examples in the nanoparticles' usage in NIR imaging (11).

Thrombus, blood clot, is the final product of the blood coagulation step in hemostasis and is formed by aggregated platelets and a mesh of cross-linked fibrin protein (12-15). Although currents effectively lyse clots and prevent tissue and organ death using exogenous plasminogen activators (PAs), these PAs may also damage normal hemostasis, which may lead to life-threatening bleeding such as intra-cerebral hemorrhage. McCarthy et al. (16) aimed to develop new thrombus-targeted fibrinolytic agents that employ the multifunctional theranostic nanomaterial to generate efficacious thrombolytic effects, while minimizing deleterious side effects (16). In this study, the authors developed thrombus targeting theranostic nanoparticles using cross-linked dextran coated iron-oxide (FeO) nanoparticles carrying imaging, therapeutic, and targeting agents. The surface of the FeO nanoparticles were functionalized with free amine groups so that imaging and targeting agents with amine reactive functional groups can be covalently attached to the surface of the nanoparticle.

To accomplish thrombus targeting, the nanoparticle was decorated with an activated factor XIII (FXIIIa)-sensitive peptide. Therapeutic ability is provided using tPA, which is covalently conjugated to nanoparticles via PEG linker (Figure 7). Commercially available carboxylic acid bearing VT680 dye is used as florescence reporter (16).

Following the synthesis of nanoparticles, the applicability of the FXIIIa-targeted thrombolytic nanoagent in the treatment of thromboembolism was demonstrated in vitro and in vivo in a murine model of arterial and venous thrombosis. Investigation of the safety profile of the nanoagent was planned to performed in another study (16).

Although it is beneficial to use fluorescence imaging, NIR imaging can be coupled with other imaging technologies such as magnetic resonance imaging and optical coherence tomography (OCT) to improve detection/imaging efficiency. In one of the most recent papers published in European Heart Journal in 2015 by Hara et al. (16), the dual imaging (NIRF-OCT) system for intravascular fibrin after stent implantation, which has a potentially clinically translatable technology, has been studied. In this study, the authors reported about diag-

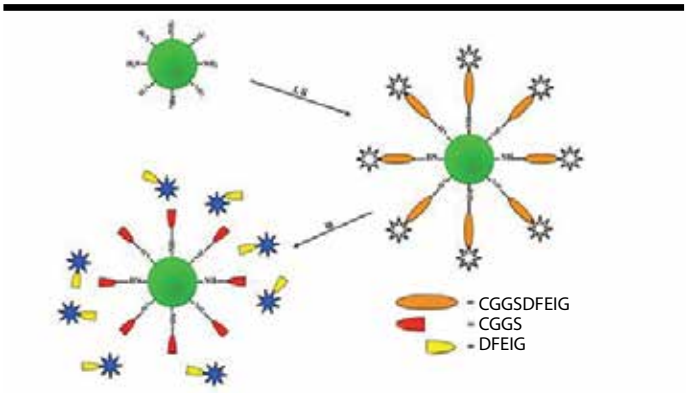

Figure 8. Modification of the polylysine graft copolymer and fluorescence activation (30)

nosis and prevention of stent thrombosis, which is a life-threatening complication of coronary artery stent implantation that occurs when a blood clot forms acutely within a stent in both bare metal stents (BMS) and drug-eluting stents (DES). OCT is a non-invasive imaging test that uses light waves to take cross-section pictures of your retina and is increasingly used for assessing stent tissue coverage as a measure of healed stents (17-21). Although OCT is increasingly used for assessing stent tissue coverage as a measure of healed stents, it cannot accurately identify whether an overlying tissue represents physiological neointima. In this study, the authors evaluated and compared fibrin deposition and persistence on BMS and DES using NIRF molecular imaging in vivo, in combination with simultaneous OCT (22).

The identification of overlying tissue represents physiological neointima visualized by near-infrared fluorescence (NIRF) molecular imaging in vivo, in combination with simultaneous OCT stent coverage (23, 24). The authors employed CyAm7 as an NIR reporter dye. Fibrin targeted imaging agent consisting of a fibrin targeting peptide and CyAm7 was prepared and validated in murine thrombosis. For in vivo studies, rabbits underwent an implantation of one BMS and DES without overlap. At Days 7 and 28, intravascular NIRF-OCT was performed following the injection of fibrin-targeted NIRF molecular imaging agent FTP11-CyAm7. Compared with BMS, DES showed greater fibrin deposition and fibrin persistence at Days 7 and 28. The results showed that the detection efficiency of unhealed stents is improved by intravascular NIRF fibrin molecular imaging techniques. A significant percentage of stents evaluated using OCT are found to be covered by fibrin, specifically in DES. Thus, there is a great possibility that such stents might remain prothrombotic. These findings indicate the specificity of clinical OCT for the evaluation and follow up of stent healing.

In another thrombosis-based study, Stein-Merlob et al. (22) NIR imaging is coupled with different imaging technologies. In this study, published in 2015 in the authors utilized high-resolution in vivo optical molecular imaging with FTP11, a NIRF fibrin-specific reporter, to investigate the in vivo inter relationships of blood accessibility to fibrin, thrombus age, thrombus neoendothelialization, and fibrinolysis in murine venous thrombosis (VT). Theranostic IVM fibrin molecular imaging strategy was developed to predict the fibrinolytic response based on fibrin accessibility of FTP11via imaging signal. NIRF microscopy showed that FTP11 fibrin binding was thrombus age-dependent. FTP11 localized to the luminal surface of early-stage VT, but only minimally to subacute VT. The authors concluded that VT fibrinolysis diminishes with thrombus age and relates to the accessibility of fibrin to blood-based fibrinolytic enzymes. Also the in vivo FTP11 fibrin accessibility signal predicts the efficacy of exogenous fibrinolysis (25).

Fibrin is the major proteinaceous component of the initial thrombus scaffold and provides a surface for thrombus propagation and eventual vessel occlusion.

As discussed above, current imaging techniques provide accurate information about the presence of thrombus and the blood flow within the vessel. However, important aspects, such as the age and anatomy, of the thrombus cannot be detected using the present methodologies. Therefore, new techniques, having huge potential, are being explored (25).

NIR dyes are valuable not only for imaging but also for the detection of enzyme activity (26-29). Konishi et al. (30) have published one recent example of this in 2015 in Circulation Journal. The authors proved that NIR imaging can be also used for the detection of enzymatic activity. This study validates a novel molecular imaging tool that enables the in vivo visualization of granzyme B activity, a major effector of cytotoxic CD8+ T lymphocytes. The authors synthesized and optimized a fluorogenic substrate capable of reporting on granzyme B activity. The substrate composed of polylysine graft copolymer and granzyme B specific peptide. The peptide having the sequence GIEFDSGGC is modified with Cy5.5-analogous (CyAI5.5B) fluorescent dye on the N terminus (Figure 8). The probe's specificity examined ex vivo in mice hearts with experimental cytotoxic CD8+-mediated myocarditis using fluorescence reflectance imaging. Granzyme B, released from CD8+ T cells, induces apoptotic death of target cells by caspase-dependent mechanisms, and a major effector of cytotoxic CD8+ T is lymphocytes. In the continuation of the study, in vivo experiments were carried for the detection of localized granzyme B activity in murine model with acute myocarditis. Fluorescent molecular tomography in conjunction with co-registered computed tomography imaging was employed for in imaging purposes. The authors confirmed that molecular imaging of granzyme B activity can visualize T cell-mediated myocardial injury and monitor the response to an anti-inflammatory intervention. Although for clinical translation there are still limitations such as the depth dependence of fluorescence imaging that prevents this method to be used on large animal or human hearts, this will be a pilot study for the future development of further novel tools that can investigate the mechanisms of immune-mediated cardiac processes, including acute cardiac transplant rejection, and evaluate the effects of therapeutic interventions (30).

A recent study published in the Journal of Biomedical Optics by Erdem et al. (31) in 2014 is another example of the detection of enzymatic activity using fluorescence as a tool (31). Authors designed and synthesized a β-lactam specific probe (off/on probe) to be able to detect one of the most commonly prescribed antibiotic β-lactam's antibiotic susceptibility and resistance in less than 30 min. For this purpose, β-lactam core of the antibiotic was modified with two identical BODIPY dyes to serve as fluorophores. When the enzyme is not introduced, two fluorophore are in close proximity and quench each other's fluorescence (off state). When the β-lactamase enzyme is introduced to the system, the β-lactam core is cleaved and fluoro-

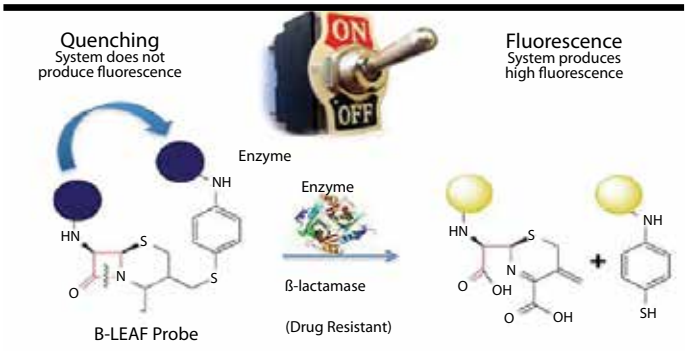

Figure 9. On/off state scheme of β-lactamase enzyme-activated fluorophore (β-LEAF) (31)

phores are separated and get way from each other. Consequently, the system gains fluorescence (on state) and fluorophores dequench. According to the quenching-dequenching, antibiotic resistance of an individual can be clarified in an easy and quick way (Figure 9).

Therapy

Besides their usage in various imaging platforms, some of the NIR imaging agents are also used as a PS in PDT applications (32-35). PDT is a promising non-invasive localized treatment modality for a diverse range of diseases, including various types of cancers, infections, and inflammatory conditions. It involves combination of light and a PS. PS is activated after treatment with light, and it causes local tissue damage via the generation of reactive oxygen species (ROS) (Figure 10). PSs that are being used in PDT are usually highly hydrophobic molecules with very low water solubility. As a result of low solubility, these molecules tend to aggregate and cause reduced ROS generation. The aforementioned cascade of events causes low PDT efficiency. Some of the PSs' general structures are shown in Figure 11 (36, 37).

In a recent review paper published by Avcı et al. (37) the overall goal of PDT is described as destroying the target tissue, while leaving healthy tissues undamaged. However, while target tissues take up PS with some selectivity, some PS also accumulates in surrounding healthy tissues. Thus, another limiting factor of PDT is that light directed at the targeted tissue site may damage adjacent healthy tissues. This prevents the use of higher PS amounts, which potentially leads to incomplete treatment responses. To solve this problem, many laboratories have been working on the development of new methods for site-specific delivery of PS such as the encapsulation of PS into drug delivery vehicles (37).

The authors also elaborated the PSs' self-assembly to form nanoparticles for PDT. To improve PS delivery to the specific site, they are often encapsulated or conjugated in/on nano-drug delivery vehicles including but not limited to liposomes, fullerosomes, and nanocells. Taken together, based on their conclusion, PDT-based activation incorporated with nanotechnology can further enhance effective drug-delivery while minimizing side-effects and is expected to be clinically applicable in the near future.

Last but not the least, in 2014, another article has been published by Spring et al. (38) to show that it is possible to selectively treat microscopic tumors using an activatable immunoconjugate utilizing

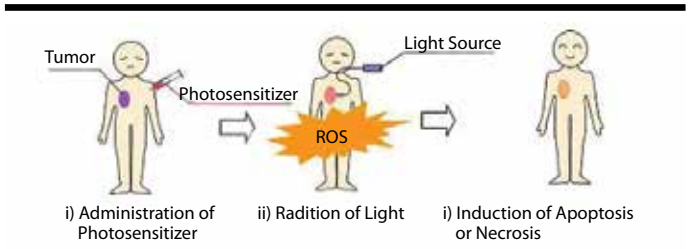

Figure 10. Profile of PDT treatment (36)

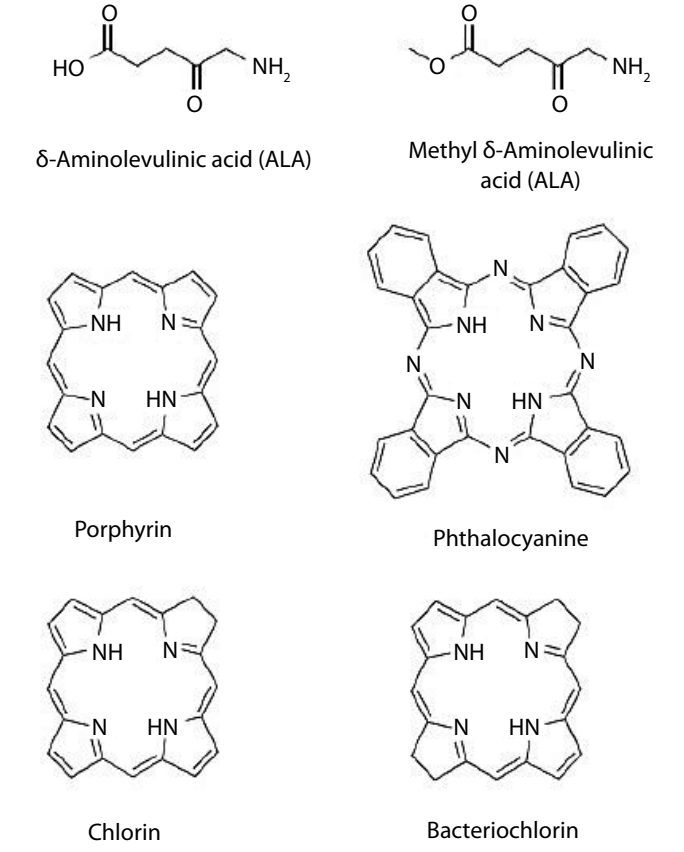

Figure 11. General structures of photosensitizers (6)

a NIR dye (38). The team chose to treat micro metastases because in standard therapies, they are not detected until the late stage of the disease. The detection is proceeded by immunoconjugate composed of self-quenching, near infrared chromophores loaded onto a cancer cell-targeting antibody. In the mentioned study, EGFR monoclonal antibody (mAb) was used as to target cancer cells overexpressing EGFR. EGFR is an important molecule for targeting cancer cells that displays elevated expression in up to 70% of EOCs and in many other carcinomas (39, 40). Benzoporphyrin derivative is a clinically-approved NIR photoactivable and cytotoxic chromophore. After conjugation with an antibody it undergoes electronic excited singlet state quenching and although fluorescence is activated as a result of lysosomal proteolysis, chromophore phototoxicity is gained after light treatment following cancer cell internalization enabling tumor-confined photocytotoxicity and the resolution of individual micrometastases. Although EGFR-targeting is the focus here, immu-

noconjugate synthesis, imaging, and taPIT can be applied to many other targets. This unique antibody-based system that couple both NIR imaging and PDT in the same platform does not only introduce a therapeutic strategy to help destroy residual drug-resistant cells but also provides a sensitive imaging method to monitor micrometastatic disease in common sites of recurrence and provides effacing the residual micro metastases limits ability to cure many cancers in standard therapies. Fluorescence microendoscopy was used to monitor immunoconjugate activation and micrometastatic disease. Tumor-targeted, activatable photoimmunotherapy' (taPIT) has been demonstrated in a mouse model of peritoneal carcinomatosis.

CONCLUSION

In conclusion, outstanding, rapid progress of great scope has put NIR dyes in the center of attention. NIR dyes' tunable chemical, photochemical, and photophysical properties with their minimal toxicity make these dyes powerful tools not only for NIR imaging in divergent fields but also for PDT of various diseases. Although NIR dyes' utility is widely accepted, the only clinically approved (FDA approved) material is indocyanine green (ICG). ICG has a quantum yield of only 0.01 in aqueous solution, which is very poor compared to other NIR dyes. Moreover, there have been reports of poor stability, rapid clearance from the liver, and cytotoxicity. It is very clear that there is a great need for new NIR dyes having high fluorescence quantum yield, improved molar absorptivity, and low cytotoxicity that could be used in clinics and/or in pre-clinical studies.

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