REVIEW

Application of Near-Infrared Dyes for Tumor Imaging, Photothermal, and Photodynamic Therapies

AHU YUAN,[1] JINHUI WU,[1] XIAOLEI TANG,[1] LILI ZHAO,[1] FENG XU,[2,3] YIQIAO HU[1]

[1]State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, P.R. China

[2]Biomedical Engineering and Biomechanics Center, Xi'an Jiaotong University, Xi'an 710049, P.R. China

[3]MOE Key Laboratory of Biomedical Information Engineering, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an 710049, P.R. China

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ABSTRACT: Near-infrared (NIR) dyes, small organic molecules that function in the NIR region, have received increasing attention in recent years as diagnostic and therapeutic agents in the field of tumor research. They have been demonstrated great successes in imaging and treating tumors both *in vitro* and *in vivo*. And their different applications in clinical practices have made rapid gains. This review primarily focuses on the progress of the application of NIR dyes in tumor imaging and therapy. In particular, advances in the use of different NIR dyes in tumor-specific imaging, photothermal, and photodynamic therapies are discussed. Limitations and prospects associated with NIR dyes in diagnostic and therapeutic application are also reviewed. © 2012 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:6–28, 2013

Keywords: cancer; drug delivery systems; nanoparticles; near-infrared spectroscopy; probe substrates; polymeric drug carrier; toxicity

INTRODUCTION

Tumor has been a major public health problem around the world. Recently, about one in four deaths results from tumor. It is projected that about 577,190 patients in the United States will die from tumor in 2012.[1,2] The high incidence rate of tumor mortality is primarily because of several elements in tumor diagnosis and therapy. First, because of the lack of effective early tumor detection, many tumors are currently detected in advanced stages. According to statistics, up to 70% of ovarian tumors become metastatic or deteriorative before diagnosis.[3] Recently, several imaging modalities, such as magnetic resonance imaging, positron emission tomography, computerized tomography, X-ray radiography, and ultrasound, are widely being used for detecting the changes of function and structure in tumor areas. However, the major challenges for these conventional imaging modalities are difficult to achieve a high contrast over nearby normal tissues and distinguish malignant tumors from benign lesions.[4–6] It is mainly due to the poor tumor affinity of conventional contrast agents. In addition, to those whose cancers are at advanced or recurrent stage, conventional clinical treatments, such as chemotherapy and radiation, may appear to be inadequate. It is mainly due to tumor resistance and severe side effects.[7,8] Both therapeutic methods make use of cytotoxic drugs or radioactive rays to destroy tumor cells. But these two conventional treatments will damage or destroy healthy tissue or cells during tumor therapy because they lack of tumor specificity. Therefore, conventional chemotherapy and radiation will induce many local or systemic side effects such as severe marrow suppression and central nervous signs. Sometimes treatments have to be discontinued for these serious side effects. Additionally, some tumor cells will become resistant during chemotherapy or radiation process. After some cycles of chemotherapeutic drugs, upregulation of some transporters such as P-glycorprotein will eject cell-killing molecules to reduce drug concentration in tumor cells, which has been a major factor for the failure of chemotherapy. Besides chemotherapy, tumor tissue or cells will

Correspondence to: Jinhui Wu (Tel.: +86-25-83596143; Fax: +86-25-83596143; E-mail: wujj@nju.edu.cn); Yiqiao Hu (Tel.: +86-25-83596143; Fax: +86-25-83596143; E-mail: huyiqiao@nju.edu.cn)

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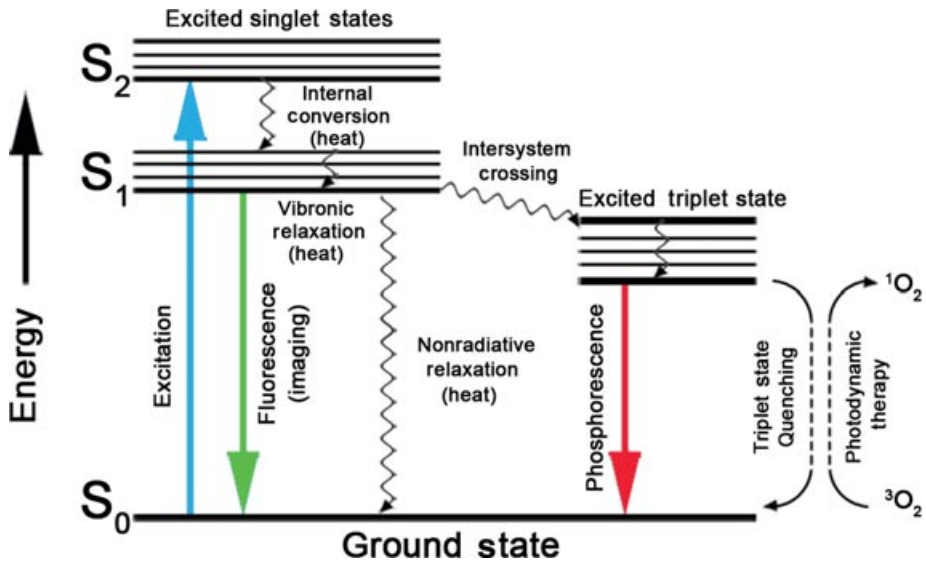


Figure 1. Energy level diagram for a photoluminescence system. Following absorption of a photon (blue arrow), several processes occur with varying probabilities. The first thing happens is relaxation to the lowest vibronic energy level of the first excited state (S1) through internal conversion or vibronic relaxation pathways (black wavy line). The excess energy is converted into heat. Then, relaxation from the lowest excited singlet state to ground state occurs, which is accompanied with emitting a photon. The process is known as fluorescence (green arrow). Some electrons in the excited singlet state can move to a lower energy excited triplet state via intersystem crossing. The latter event ultimately results in emitting a photon through phosphorescence (red arrow). Triplet oxygen, the ground state of oxygen molecule, is a very effective quencher for fluorophores in the excited triplet state. It can be excited to reactive singlet state (right-hand dotted line), producing phototoxic effect to living cells.

also become resistant to radiation. As reported, high-energy radioactive rays can destroy most of tumor cells. But some survived tumor cells will become cancer stem cells, which are insensitive to radiation therapy. And these cancer stem cells are considered as seeds for tumor recurrence.[9]

Near-infrared dyes, as promising imaging and therapeutic agents, have been of great interests in detecting and treating tumors recently. They can absorb NIR light with a specific wavelength to reach an excited singlet state. Part of the energy of the excited singlet state would be dissipated in the form of light with a longer wavelength, called fluorescence. Therefore, NIR dyes can be applied for *in vivo* tumor imaging effectively. And it has high specificity because targeted NIR molecules can distinguish the molecular changes between tumor and normal tissues. Furthermore, NIR imaging shows high sensitivity owing to extremely low absorption and autofluorescence from organic tissue in the NIR spectral range, which can minimize background interference and improve tissue penetration.[10]

In addition, some energy of the excited singlet state can be transited through vibronic relaxation or other nonradiative transitions pathways, which will be converted into heat. If the rate of heat production within the tissue can exceed that of tissue heat dissipation, the temperature of tissue would increase gradually. When temperature reaches up to 41.5°C, tumor cellular cytotoxicity occurs. And temperatures above 43°C can induce vascular destruction within tumor tissue. Therefore, NIR dyes can also be utilized as promising theranostic agents for photothermal therapy (PTT) while detecting tumors. Apart from the above-mentioned two kinds of energy transition way, the excited singlet state can move to a lower-energy-excited triplet state via intersystem crossing. In the excited triplet state, NIR dyes can induce reactive species generation, for example, free radical or reactive singlet oxygen. They induce oxidation reaction with nearby biomacromolecules and destruct organic tissues effectively. In which, generated singlet oxygen is more responsible for the destruction of targeted tissue. Thus, NIR dyes could also act as excellent photodynamic agents (Fig. 1). Compared with conventional methods, photothermal and photodynamic therapies have different therapeutic mechanism and modalities. First, targeted photothermal or photodynamic agents accumulate in tumor sites actively, and the tumor areas are imaged and located distinctly. Then, clear tumor imaging will guide laser treatment to the tumor site alone. And heat energy and singlet oxygen produced by therapeutic agents will destroy adjacent tumor cells in cancer areas.[11] Therefore, these

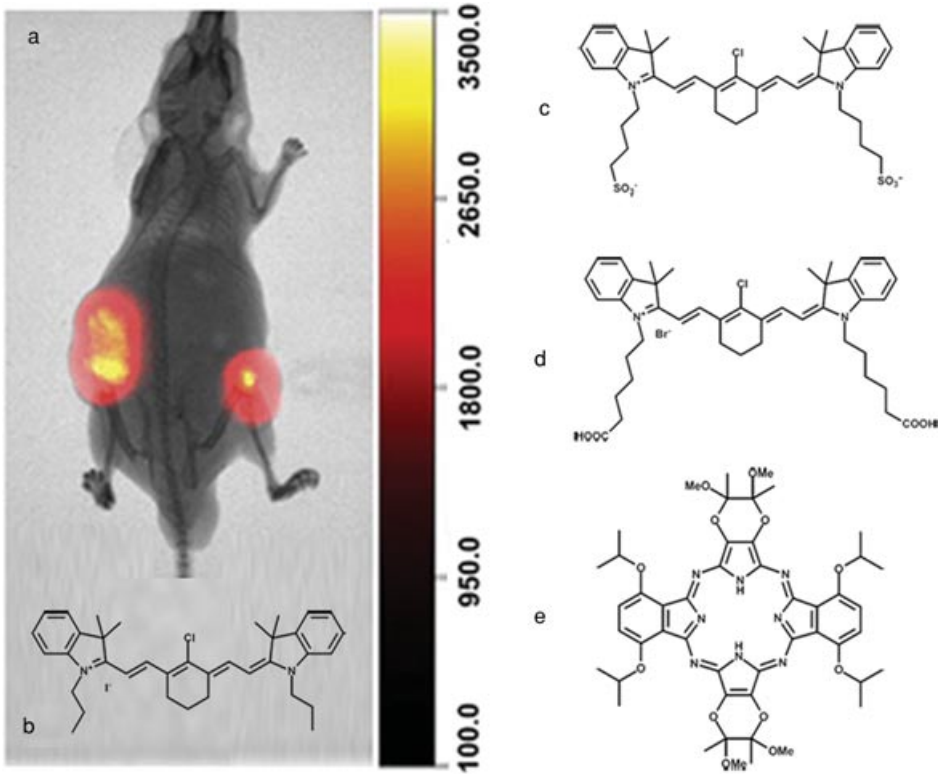


Figure 2. Tumor targeting and imaging by free NIR dyes. Merged X-ray and NIR fluorescent imaging of MCF-7 tumors in athymic nude mouse with cyanine dye IR-780 iodide (a).[15] Chemical structures of NIR dyes with tumor affinity: IR-780 iodide (b), IR-808 (c), IR-783 (d), and Pz-247 (e).

two therapies can obviously avoid drug resistance and decrease severe side effects.[12–14] Recently, they have already been novel alternative strategies to tumor treatment.

This review is focusing on the recent progress of versatile NIR dyes in tumor research. These progresses have broadened existing concepts of tumor diagnosis therapeutics. In particular, advances and limitations of NIR dyes in tumor imaging, photothermal, and photodynamic therapies are extensively reviewed.

NIR DYES FOR TUMORS IMAGING

Specific optical tumor imaging *in vivo* holds significant promise for early tumor diagnosis and assessing tumor response during treatment. Noninvasive optical imaging methods are of great interest both in clinical or preclinical practices. In these diagnostic procedures, target tissues are illuminated by fluorescent or radiant signals after administration of imaging agents, which can effectively distinguish tumors from normal tissues. Among them, near-infrared (NIR) fluorescence tumor imaging exhibits great potential because of the high tissue penetration depth and low tissue autofluorescence interference in the NIR spectrum window. After an intravenous injection, these tumor probes will show bright fluorescence signal in tumor region upon excitation. Thus, NIR fluorescence imaging emerges as an excellent modality for imaging pathological changes, especially tumors, in a noninvasive and real-time way. The key of tumor imaging is to improve specificity to distinguish tumors from the normal tissues. In particular, improving the tumor to background ratio (T/B) value attracts much attention from different research groups. To date, several strategies including targeted free NIR dyes, NIR dye conjugates, and activatable NIR dyes have been conducted to improve tumor signal, decrease background signal, or both.

Free Dye for Tumor-Targeted Imaging

Near-infrared dyes reaching a high T/B value are suitable for *in vivo* tumor imaging. One of the strategies is to search and develop free dyes with ability of accumulating in tumor tissue actively. There are at least two classes of NIR dyes, cyanine dyes and porphyrazine derivatives, satisfying this requirement. First, Zhang et al.[15] reported a cyanine dye, IR-780 iodide, an extraordinary hydrophobic dye with excellent optical property, for tumor-targeted imaging (Figs. 2a and 2b). T/B value of IR-780 iodide *in vivo*

increased from 2 at day 1 to 7 at day 20. However, the too high hydrophobicity of IR-780 iodide resulted in extremely low solubility in pharmaceutically acceptable solvents, which was adverse for administration and inhibited the widely application potentially.[16] Yang et al.[17] reported that IR-783 (Fig. 2c), a derivative of IR-780 iodide with better hydrophilicity, could also selectively accumulate in tumor tissues. It was found that IR-783 could detect xenograft and metastasizing tumors. Similar to this, IR-808 was also found to accumulate in tumor tissues actively. IR-808 was also less hydrophobic compared with IR-780 iodide because of the addition of two carboxyl groups (Fig. 2d).[18] Another advantage of IR-808 was its lower systemic toxicity compared with IR-780 iodide. In a period of 7 days, mice treated by up to 150 mg/kg IR-808 via intravenous injection showed no obvious acute toxicity. The other group of NIR dyes for targeting tumor and imaging is porphyrazine derivatives. Porphyrazine 247(Pz-247, Fig. 2e), with above 700 nm fluorescent emission, exhibited preferential accumulation in tumor cells with low background signal. It had a macrocycle core, which was different from the structure of cyanine dyes. And it was demonstrated to associate with the hydrophobic core of low-density lipoprotein and get into tumor cells specifically through receptor-mediated endocytosis.[19]

These targeted free NIR dyes showed some advantages, such as convenience, in tumor imaging *in vivo*. In addition, these free NIR dyes are not confined to several specific tumor types. They can be selectively taken up by a broad spectrum of tumor types. However, there still are some obstacles for their improved clinical application, for example, poor solubility and systemic toxicity. Additionally, some hydrophobic NIR dyes would accumulate in normal organs such as lung and testicle. Therefore, safety of these imaging agents still needs further studies. Overall, efforts in searching ideal NIR dyes with sufficient solubility, low toxicity, and importantly enough tumor specificity will significantly promote the development process of tumor imaging.

NIR Dyes Conjugated with Targeting Ligands for Tumor Imaging

A small molecular probe with tumor specificity is curial for specific tumor detection. However, most NIR dyes, such as indocyanine green (ICG), are with no or limited tumor targeting property, which greatly restrict their application in tumor imaging. Therefore, different strategies are designed to improve the affinity between tumor cells and NIR dyes. Chemical conjugation of NIR dyes to tumor-specific ligands was the most common strategy. These ligands include arginine–glycine–aspartate (RGD) antibodies, whose receptors overexpressed in specific tumor cells. The complex made of NIR dyes and targeted ligands could function as tumor-specific imaging probes and increase the T/B value *in vivo* imaging.

Chen et al.[20] synthesized a NIR dye conjugate, Cy5.5–RGD for target tumor imaging. Integrin is a family of transmembrane cell adhesion receptors overexpressing on many tumor cell surface and its binding factors mostly contain RGD triad. Therefore, the small peptide RGD was developed as a special ligand for tumor targeting[21]. Results showed Cy5.5–RGD could target integrin $\alpha_V\beta_3$-positive U87MG glioblastoma model effectively. To further improve the targeting ability, a cyclodecapeptide platform named RAFT (regioselectively addressable functionalized template) was designed. RAFT, having two spatially independent functional domains, can be covalently and specifically modified with four RGD molecules and a NIR dye simultaneously (Fig. 3b) such as Cy5. Jin et al.[22] reported that Cy5–RAFT–c(RGD)$_4$ could specifically target $\alpha_V\beta_3$-integrin-overexpressed HEK293 (Human Embryonic Kidney 293) tumor cells and did not accumulate in the $\alpha_V\beta_3$-negative HEK293 xenografts. Furthermore, the T/B value of Cy5–RAFT–c(RGD)$_4$ (Figs. 3b and 3d) was significantly higher than that of Cy5–cRGD (Figs. 3a and 3c). And other reports[23,24] further confirmed the amazing phenomenon. These results might be due to conjugation of four RGD molecules to one probe, greatly improving the probability of probes binding to specific receptors on tumor cells.

In addition, the overexpression of some protein receptors such as epidermal growth factor receptor (EGFR) is observed in many tumors.[25] Therefore, epidermal growth factor (EGF)-modified NIR dye could specifically image tumor cells by targeting EGFR. Ke et al.[26] reported a EGF-modified NIR dye (EGF–Cy5.5) whose fluorescence signal was clearly visualized in MDA-MB-468 (EGFR-positive) tumors but not in MDA-MB-435 (EGFR-negative) tumors. Meanwhile, the fluorescence signal in MDA-MB-468 tumor region could be specifically blocked by the preinjection of C225, a specific monoclonal antibody (mAb) of EGFR. Similar to EGF, C225–Cy5.5 conjugate was also developed and served as an excellent diagnostic modality.[27] Besides EGFR, there are many other receptors, such as FGFR (fibroblast growth factor receptor) and VEGFR (vascular endothelial growth factor receptor), overexpressed on tumor cells or tumor-associated endothelial cells. Their ligands can also be utilized to increase the tumor specificity of NIR dyes.[28,29]

To date, this general and effective approach for improving tumor specificity by conjugating NIR dyes to tumor-targeted ligands has achieved many important improvements. Apart from molecular targeting, these macromolecular ligands will also improve the half-life and distribution of the NIR dyes. Overall, conjugating NIR dyes to ligands can make the dyes function

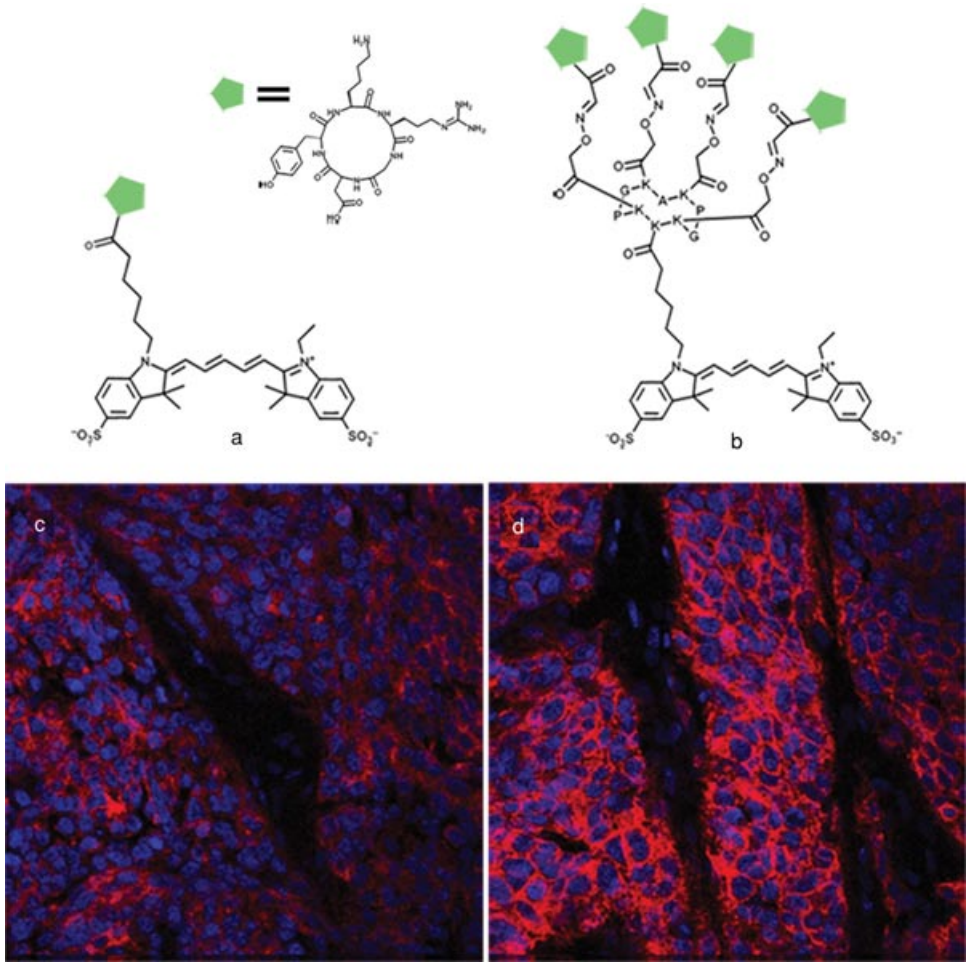


Figure 3. Schematic structures of Cy5-labeled cRGD (a) and RAFT-c $(RGD)_4$ (b). Confocal laser scanning microscopic images of $\alpha_V\beta_3$-integrin-overexpressed HEK293 tumors administrated by Cy5-labeled cRGD (c) and RAFT-c $(RGD)_4$ (d). Signal from Cy5 was red, and paraformaldehyde-fixed tumor tissue was incubated with Hoechst 33342 for nuclear staining (blue).[22]

as tumor-specific imaging probes and increase the T/B value. These bring NIR dyes widely application in tumor imaging. However, there still exist some drawbacks needed to be optimized, for example, high cost of some targeting ligands and the complicated process of modification. In addition, it is also important to avoid breaking the structures and decreasing the binding affinity of targeted ligands during chemical modification process.

Nanoparticles-Mediated NIR Probe for Tumor Imaging

Another strategy for increasing T/B values of NIR dye is nanoparticles. With the great advancement of nanotechnology, nanoparticles-mediated NIR probes (nanoprobes) have shown great potential to overcome disadvantages, such as poor solubility and lack of tumor targeting ability, of free NIR dyes. Nanoparticles containing NIR dyes could accumulate in tumors tissue or cells through passive[30] or active targeting approaches. Many dye-conjugated or dye-encapsulated nanoparticles, for example, inorganic nanoparticles, liposomes, and polymer nanoparticles, have been developed and evaluated as tumor imaging agents.[31,32] For instance, Reul et al.[33] covalently labeled PLGA with an NIR dye, DY-700, and obtained NIR fluorescent nanoparticles successfully. They significantly reduce the toxicity and enhanced the solubility of NIR dyes. Even more sufficient T/B value can be obtained as outstanding tumor-specific probes compared with free dyes.[31–33] In addition, because of protection of nanoparticles, nanoprobes show much better photostability than free NIR dyes. However, some limitations of nanoparticles, such as the potential long-term systemic toxicity of nanomaterials, the difficulties in the large-scale preparation, and the dubious stability after lyophilization, still limit the clinical application of these nanoprobes.[34–36] Therefore, fabrication of biodegradable and nontoxic and more specific nanoprobes will be one of the orientations for nanoparticle-based NIR dye tumor imaging. In

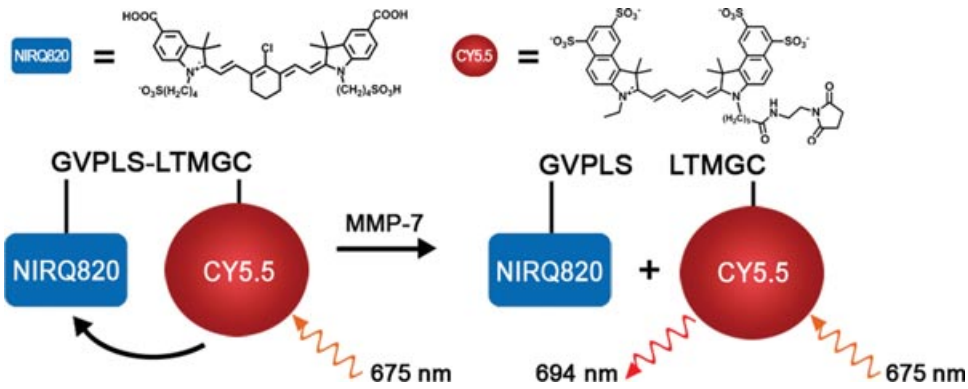


Figure 4. Schematic structures of MMP-7 activatable probes. Because of the proximity between Cy5.5 and NIRQ820, fluorescence quenching occurs and almost no fluorescent can be detected in the inactive state. After specific enzyme cleavage of substrate, Cy5.5 molecules are released away from the NIRQ820 and brightly fluoresce.

which, biomacromolecules such as albumin, which have been approved by US Food and Drug Administration (FDA) for intravenous administration, will be promising carriers for developing nanoprobes. It is mainly because of their superior biocompatibility and biodegradability.

Activatable NIR Fluorescent Probes for Tumor Imaging

Conventional fluorescent imaging probes emit fluorescence signals regardless of their environment, either diseased or normal tissues, which results in high background signal. And these conventional probes are vividly termed "always on" probes. To decrease background signal, activatable probes are designed, which is "turned off" under normal condition, whereas can be "turned on" under certain diseased conditions. When in inactive or quenched state, these activatable probes showed no or little fluorescence. After specific activation at the target site, such as specific enzymatic digestion or acidification in tumor tissues or cells, the activatable probes will be capable of emitting strong fluorescent signal upon excitation. So these activatable probes, including general and targeted activatable probes, became superior probes with minimal background interference, which in turn improved T/B value and tumor specificity.

General activatable probes are small organic probes, which exist in inactive state until activated by specific enzymatic cleavage. To date, various general activatable probes have been developed to detect and image different tumors *in vivo*. They showed low background fluorescence signal compared with conventional probes. For instance, Pham et al.[37] reported a matrix metalloproteinase 7 (MMP-7)-sensitive activatable probe to detect tumor. MMP-7 is an representative protease overexpressed in tumor tissue and has been widely confirmed to be related with tumor invasion and metastasis. This activatable probe was synthesized by attaching a quencher (NIRQ820) and fluorescent probe (Cy5.5) to both ends of an MMP-7 substrate. Because of their proximity to each other, NIRQ820 became an efficient quencher for Cy5.5 (Fig. 4). *In vitro* studies showed that the probe could be specifically cleaved by MMP-7, and then the fluorescent signal increased by sevenfold. However, some disadvantages of these low-molecular-weight activatable probes appeared with the continual studies. Most of these small molecules lack of tumor specificity and they would distribute in the whole body. And some enzymes used as model target were not specific for tumor.[38–40] They might appear in normal or inflammatory tissues, which would bring up background signal or even false-positive results during detecting tumors. Therefore, selecting more specific enzymes as probe activator has attracted much attention of many researchers.

Another strategy for improving T/B value is to promote tumor region signal and decrease background signal simultaneously. And the main approach for enhancing tumor accumulation based on EPR effect or molecular targeting pathways of macromolecule/nanoparticles. Recently, many macromolecular/nanoparticle tumor cell/tissue-targeted activatable probes have been designed.[41,42] After tumor accumulation, these targeted activatable probes will be activated by specific stimulus, such as enzymes or low pH, and image the tumor profile. On the contrary, the background fluorescence is extremely low in normal tissue or cells because of the lack of stimulus. Tung et al.[42] reported an enzyme-sensitive activatable probe in which Cy5.5 molecules were conjugated to a synthetic long circulating copolymer, containing cleavable substrate for cathepsin D. Cathepsin D is a protease and overexpressed in tumors. High levels of Cathepsin D in tumor cells is demonstrated to be associated with greater invasiveness.[43] The polymer based activatable probe accumulated in tumor tissues via EPR effect. A clearly detectable NIR fluorescence

signal could be observed from cathepsin-D-positive tumors in live animals, which emitted much higher fluorescence than the cathepsin-D-negative tumors significantly.

In addition, various biomolecules, such as antibodies and RGD, attached to activatable probes are also conducted for improved T/B value. For instance, trastuzumab, a mAb against the EGFR, could be conjugated with activatable probes for tumor specificity. Ogawa et al.[44] selected the TAMRA (tetramethylrhodamine, fluorophore)–QSY7 (quencher) pair and conjugated them to trastuzumab (Traz–TM–QSY7) (Fig. 5a). After receptor-mediated internalization, trastuzumab was generally cut into amino acids or small peptides by oxidation, proteases, and pH-assisted degradation in lysosomes. Subsequently, fluorophores were activated because quenchers were separated. Traz–TM–QSY7 was found to successfully detect small tumors overexpressing EGFR. In addition, targeted pH-activatable probes could also be used for improved tumor specificity. Lee et al.[45] developed a RGD-mediated pH-sensitive activatable probe. After reaching lysosomes with pH < 5, the probes became brightly fluorescent and imaged tumor effectively. Similar to this, Urano et al.[46] established trastuzumab-conjugated activatable pH-sensitive fluorescence probe (Fig. 5b) and demonstrated efficient tumor imaging.

Through passive or active accumulation, more targeted activatable probe molecules selectively distribute in tumor tissues or cells. After that, these targeted activatable probes will be activated by specific enzymes or lower pH in tumor cells or interstitium. Because of the significant tumor sensitivity and specificity and low background signal before activation, targeted activatable probes will highlight their potential as valuable agents in detecting tumors. During the process of conjugation, maintaining the highly tumor specificity of targeted ligands (e.g., mAbs) is very important for developing targeted activatable probes. Additionally, the high cost of mAbs may be another potential barrier.

Clinical Use of NIR Dyes in Tumor Imaging

To date, some NIR dyes have been applied in clinics for detecting tumor margins or microcancer lesions. For instance, 5-aminolevulinic acid (5-ALA, Glionlan™, Medac GmbH, Germany) has been approved in Germany for distinguishing malignant glioma from normal brain tissue.[47] 5-ALA is an endogenic precursor of protoporphyrin IX (PpIX), which emits red fluorescence and can be observed by naked eyes. The biosynthesis from 5-ALA to PpIX involves many steps, among which protoporphyrinogen oxidase is a key enzyme controlling the whole reaction. Because of the overexpression of protoporphyrinogen oxidase in tumor cells, after oral administration of 5-ALA, the resulted PpIX accumulates in malignant glioma as compared with normal brain tissue.[48] Thus, 5-ALA has been applied for distinguishing tumor margins and precise resection of malignant glioma. Additionally, another NIR dye, IRDye800CW, conjugating with antibody targeting VEGF–bevacizumab, has completed preclinical toxicity studies and is currently undergoing clinical trial for early cancer detection.[49] Table 1 lists representative NIR dyes used for tumor imaging in clinics.

NIR DYES ACTING AS THERANOSTIC AGENTS IN PTT

Photothermal therapy, a noninvasive approach to treat various medical conditions, has been widely investigated in tumor therapy. It destroys tumor cells by generating heat within a tumor by absorbing specific light in the NIR region. Accumulation of NIR absorbing material, such as gold nanoshells,[55] carbon nanotubes,[56] and NIR dyes,[57,58] in tumor tissue can enhance the efficiency of PTT significantly.[59,60] It is mainly because these materials could convert energy of light into heat effectively. As photothermal agents, NIR dyes have been paid more and more attention recently. It may be because of the two principal advantages for NIR dyes over others: (1) NIR dyes can image tumor *in vivo* and guide photothermal therapeutic process, which is called theranostics; and (2) NIR dyes, such as ICG, show less long-term toxicity compared with inorganic photothermal agents such as gold nanoshells.[61] To date, NIR-dyes-based PTT has achieved highly promising results in several animal models. Therefore, more and more researchers focus on developing different platforms for the photothermal application of NIR dyes.

Free NIR Dyes Used in Tumor PTT

At first, free NIR dyes have been directly utilized as photothermal agents to induce thermal injury.[62–65] As FDA-approved commercial imaging agent, ICG was safe and convenient enough during the PTT process. Shafirstein et al.[63] evaluated the potential benefit of using free ICG/NIR laser therapy to suppress tumor growth via thermal injury. *In vivo* results showed that the laser/ICG-treated group showed an obvious reduction in tumor volume compared with the laser/saline group. However, ICG shows extremely short half-life of about 3 min.[41] After intravenous administration, irradiation should be conducted in 1 min. Otherwise, ICG had little tumor distribution and would be removed from vessel in tumor region rapidly. Laser irradiation with high power density up to 433 W/cm^2 was utilized to increase the tumor temperature to above 55°C for PTT. The very high power density used for treating tumors always induces large-scale damage of normal tissues around tumors.[66] Meanwhile, the

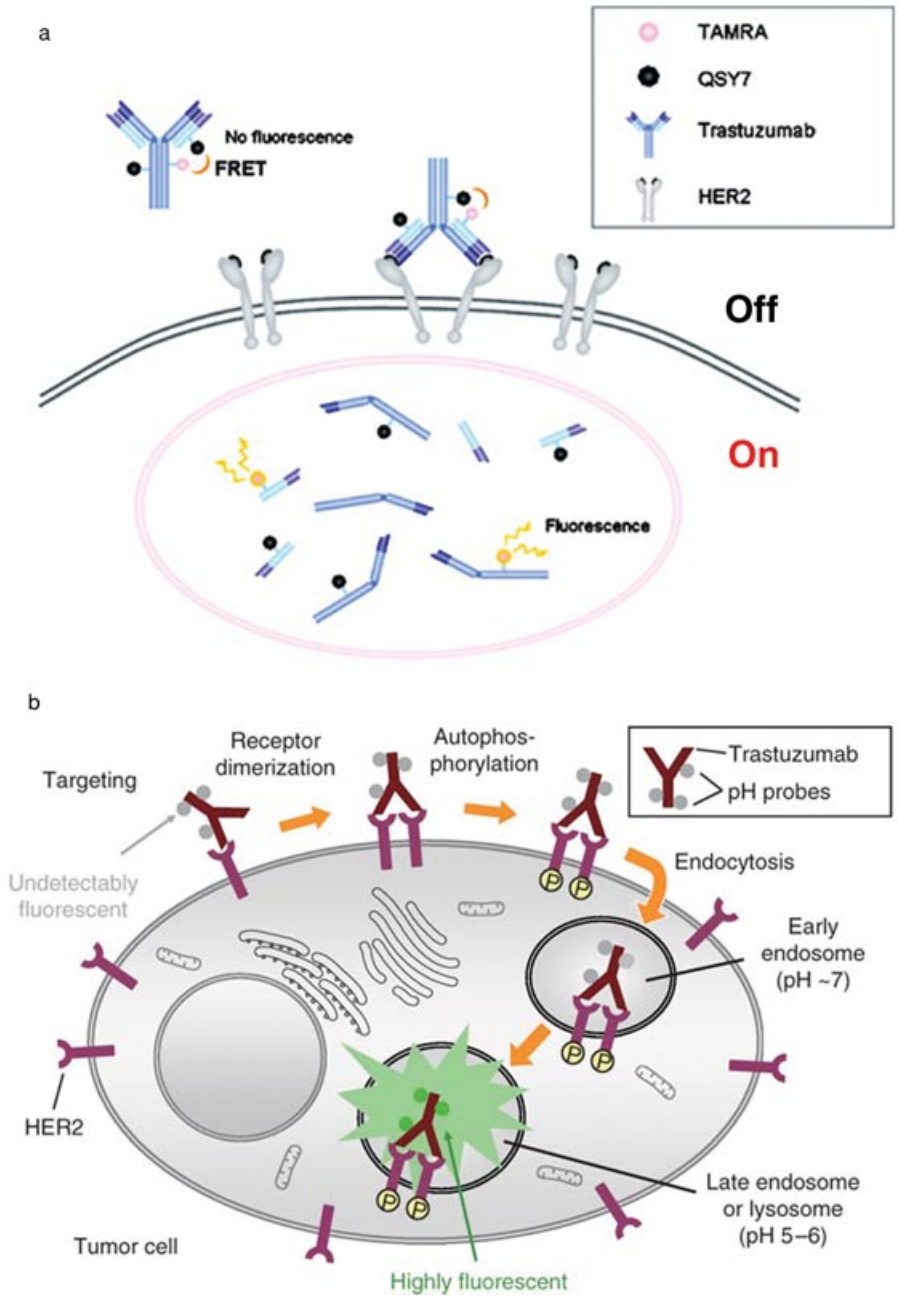
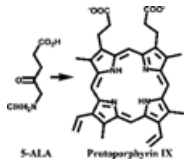
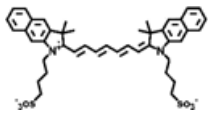
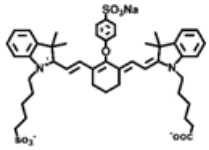


Figure 5. Schematic illustration of targeted activatable probes. Antibody trastuzumab-based targeted activatable probe Traz–TM–QSY7 is quenched because of the proximity between TAMRA (fluorophore) and QSY7 (quencher) outside of the cells. When it binds to trastuzumab receptor and is taken up by tumor cells, it is disassembled within the cell and dequenching occurs (a).[44] A targeted activatable probe consists of pH-sensitive fluorescence and antibody, which can selectively detect tumor. The probe has no fluorescent signal when outside of the tumor cells. Following internalization via antibody-mediated endocytosis, it migrates to late endosomes or lysosomes. In which, acidic pH can activate the probe and makes it highly fluorescent (b).[46]

conduction of PTT just 1 min after administration is easily out of control. Both these requirements are not conducive for clinical photothermal therapeutic application.

Although showing potential for PTT, free NIR dyes suffer from several major deficiencies needed to be addressed. First of all, most of these free NIR dyes are not stable in aqueous solutions and they are concentration-dependent aggregated.[67] In addition, some NIR dyes show insufficient tumor accumulation during PTT. If there are other NIR dyes with appropriate half-life and sufficient tumor specificity, free

Table 1. Summary of Representative NIR Dyes used for Detecting Tumor

Drug	Imaging Agent	Structure	Application	State	References
Glionlan	5-ALA-induced PpIX		Malignant gliomas imaging	Approved in Germany	47,50
ICG	ICG		Microcancer imaging and breast cancer-assisted sentinel lymph node mapping	Phase 1	51–53
Bevacizumab–IRDye800CW conjugate	IDRye800CW		Breast cancer margin imaging	Phase 1	49,54

NIR-dyes-based PTT would be more convenient and effective. As reported, some new NIR dyes, such as IR-780 iodide and IR-808, accumulate in tumor tissues after administration. Recently, they have been utilized for tumor imaging successfully.[15,18] Additionally, systemic toxicity of these NIR dyes determines whether they can be applied in PTT securely. For example, IR-780 iodide shows little toxicity when administrated in low dose for imaging,[15,68] whereas high dose for PTT shows sever acute toxicity and induces quick death of mice. With such limitation, the therapeutic application of these toxic NIR dyes was strictly limited. Fortunately, IR-808, a derivative of IR-780 iodide, shows much lower toxicity compared with IR-780 iodide. Consequently, developing more low toxic and tumor-specific NIR dyes will further promote the application of free NIR dye in PTT.

Encapsulated NIR Dyes for Theranostic PTT

Because of great potential for tumor PTT, novel drug delivery technologies have been applied to overcome deficiencies of free NIR dyes. Nanoparticle-based theranostic agent has been widely demonstrated for improving different drawbacks of small drugs and imaging agents.[69–71] Theranostics, derived from "therapy" and "diagnostics," refers to combining a diagnostics and a consequent therapy based on the diagnostic results. Most nanoparticles-based PTT relies on the accumulation of nanoparticles in tumor tissue via different mechanisms. One pathway relies on the passive accumulation in tumor via the leaky tumor vasculature. In addition to passive accumulation, nanoparticles conjugating targeted ligands could be selectively delivered to surface receptors overexpressing on tumor cells. It has been a promising strategy for improving clinical application of NIR-dyes-mediated PTT. IR-780 iodide is a hydrophobic cyanine dye with peak absorption at 780 nm, and it is virtually insoluble in all pharmaceutically acceptable solvents.[15] This dye was encapsulated in to PEG–PCL(Poly(ethylene glycol)-Polycaprolactone) polymer nanoparticles (IR-780 micelles), having dual functions for tumor imaging *in vivo* and imaging-assisted PTT.[72] The formed theranostic agent obviously improved the solubility and stability of free IR-780 iodide. They showed almost no toxicity during diagnosis and therapy such as reduced bodyweight in mice treated by IR-780 micelles. Thus, IR-780 micelles can detect tumors and guide the delivery of laser irradiation. Importantly, IR-780 micelles showed improved efficiency in photothermal ablation upon irradiation compared with the group received laser irradiation only. Lovell et al.[73] reported a more complicated and versatile theranostic agent, used as a multimodal biophotonic contrast agent. Porphysome was self-assembled from phospholipid porphyrin conjugates, exhibiting liposome-like nanostructure. Porphysome was used as an excellent activatable fluorescent probe *in vivo* first. After detecting the tumor successfully, porphysome could also be used as a photothermal agent for PTT. In addition, porphysome surprisingly showed minimal acute toxicity in mice with intravenous doses up to 1000 mg/kg.

Passive nanoparticles with appropriate size and surface chemistry could escape from reticuloendothelial system and accumulate in the tumor tissues selectively. Recently, many passive NIR dyes containing nanoparticle theranostic agents for PTT have been developed, and they also show versatile properties in both diagnostic and therapeutic fields. However, sometimes lack of tumor cell specificity might decrease the efficiency of these passive nanoparticles entering into tumor cells. And a large amount of passive nanoparticles would locate outside of tumor cells.

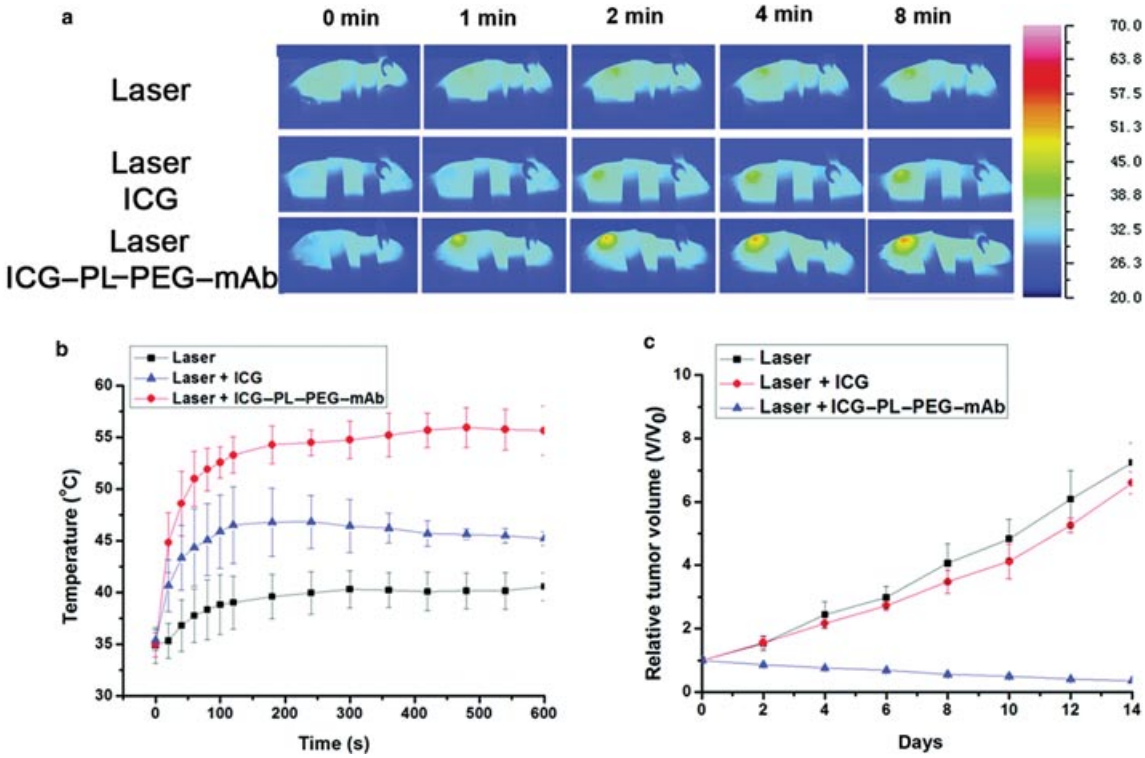


Figure 6. Photothermal treatment using ICG and ICG–PL–PEG–mAb. Thermography of mice bearing U87-MG tumors administrated by PBS, ICG, and ICG-PL-PEG-mAb upon irradiation (a). Temperature of the irradiated area increased with irradiation time ($n = 3$) (b). Tumor growth curves of U87-MG tumor ($n = 3$), laser/ICG–PL–PEG–mAb-treated group showed complete suppression of tumor growth compared with other two therapeutic groups (c).[58]

Thus, it partly limited the photothermal therapeutic efficiency to specific tumor cells.

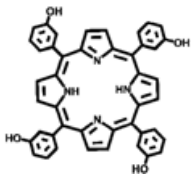
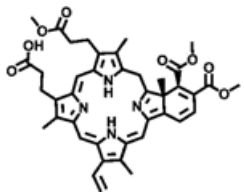
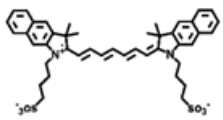
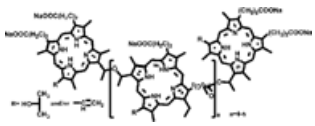
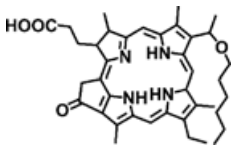
In addition to passive tumor-targeting approach, active tumor targeting was also conducted to enhance tumor specificity. This process is more reliable and specific, employing antibodies or other targeting ligands for specific tumor receptors, which were always overexpressed on the surface of tumor cells. Yu et al.[74] reported an ICG-containing active targeting nanocapsule, coated by EGFR antibodies via electrostatic conjugation. Active targeting nanocapsules could bind to EGFR-positive tumor cells. Thus, nanocapsules containing ICG successfully accumulated in tumor tissues capitalizing on both enhanced permeability and retention (EPR) effect and active targeting approach. Significant PTT efficiency was observed compared with free ICG. Similar to this, folic acid (FA) and integrin $\alpha_V\beta_3$ mAb were conjugated to the surface of ICG-loaded PL–PEG (Phospholipid-Poly(ethylene glycol)) nanoparticles, mediating the internalization of photothermal agent into targeted tumor cells successfully.[57] These active targeted nanoparticles showed complete suppression of tumor growth after irradiation (Fig. 6).

Compared with passive nanoparticles, conjugation of different targeted ligands to nanoparticles largely improved the affinity with specific tumor cells. By administration of these active targeted theranostic agents, fluorescent imaging can be applied to identify the precise location and size of tumors. Subsequently, precise tumor imaging will guide NIR laser treatment more accurately to avoid the thermal damage to surrounding normal tissues. Furthermore, after PTT, therapeutic effectiveness can be intuitively evaluated by the theranostic-agents-assisted diagnosis. The combination of diagnosis and therapy can significantly simplify the therapeutic procedure, shorten the therapeutic period, and more importantly make PTT more efficient. Although these theranostic agents hold tremendous potential in both diagnosis and imaging-assisted PTT, there are still a few minor problems needed to be addressed. First, extremely low loading of NIR dyes into nanoparticles are widespread in many studies.[57,58,72] Additional researches of potential toxicities of these novel photothermal complexes need further study as this platform transfers into clinical practice.

NIR DYES ACTING AS THERANOSTIC AGENTS IN PHOTODYNAMIC THERAPY

Photodynamic therapy (PDT), an emerging therapeutic modality, applies photosensitizers and light irradiation to destroy tumor tissues.[75] With specific

Table 2. Representative Photosensitizers Currently used in Tumor Ablation

Drug	Photosensitizer Substance	Structure	Application	State	References
Foscan	m-THPC		PDT for head and neck tumors, prostate and pancreatic tumors	Approved in Europe	77
Visudyne	Vertepofin		PDT for basal cell carcinoma	Approved in USA	78
ICG	ICG		PDT for choroidal melanoma	Phase 4	79–81
Photofrin	HPD		PDT for cervical, gastric cancer, brain tumors	Approved in Canada and USA	82
HPPH	HPPH		PDT for lung cancer, head and neck cancer	Phases 1 and 2	83

HPPH, 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a.

wavelength light exciting, the photosensitizers can interact with molecular oxygen and generate cytotoxic singlet oxygen or oxygen radicals to kill tumor cells.[76] All NIR dyes, used as theranostic photosensitizers, could image tumor conveniently before PDT. The combination of diagnosis and PDT can precisely determine the location and area of tumor and significantly enhance therapeutic effect. The first approved photosensitizer, Photofrin(Pinnacle Biologics, Inc., Bannockburn, England) in 1993 for the prophylactic treatment of bladder tumor in Canada, was the milestone of PDT, and also opened the door for wider application of PDT in tumor therapy. Photofrin is a complicated mixture of oligomeric porphyrins. It is approved for the treatment of early-stage and advanced lung cancers, esophagus cancers, and other cancers in different countries. To date, many other photosensitizers, such as benzoporphyrin derivative monoacid ring A(BPD-MA) and meta-tetrahydroxyphenylchlorin (m-THPC, Foscan, Biolitec Pharma ltd, Dublin, Ireland), have also been applied widely in PDT (Table 2). However, there still exist three major drawbacks for PDT, limiting its widely clinical application in treating various tumors. First is that photosensitizers widely used in clinics recently generally absorb light in the visible red range, for example, 630 nm for Photofrin. Light in this region penetrated into tissues only within a few millimeters, making them unsuitable for treating deep-seated tumors. Second, most photosensitizers exhibit limited tumor specificity, thus resulting in limited PDT efficacy. Third, after administration, some photosensitizer molecules locate in normal tissues, especially skin, which causes long-lasting skin photosensitivity upon exposed to sunlight. Subsequently, patients treated by photosensitizers have to avoid sunlight for more than 1 month. Therefore, there is an urgency for developing new photosensitizers to overcome these limitations for wide and effective application of PDT.

Developing Photosensitizers with Longer Wavelength of Maximum Absorption (λ_{max})

Penetration depth of light is defined as the depth at which the intensity of the light inside the tissue falls to about 37% of its original value at the surface.[84] For a given tissue, penetration depth will generally be

a function of wavelength of the incident light. Light at a wavelength of 630–660 nm will penetrate tumor tissue to a depth of less than 10 mm. And light with longer λ_{max}, for example, 700–800 nm, will penetrate to a depth of about 10 mm or over.[85,86] The deeper tissue penetration makes it skillful for the treatment of larger and deeper solid tumors. To achieve better therapeutic efficiency, excellent photosensitizers with longer λ_{max} are commonly developed for PDT. Mitsunaga et al.[87] reported that a NIR phthalocyanine dye, IR-700, was selected as an admirable photosensitizer to kill tumor with a volume of 50 mm^3 because IR-700 has a λ_{max} of 689 nm, which is longer than that of the most commonly used photosensitizers. Lutetium texaphyrin, a water-soluble photosensitizer with absorption maximum of 732 nm, with deeper tissue penetrating ability, had significant efficacy in suppressing both moderate tumors ($40 \pm 14\ mm^3$) and larger tumors ($147 \pm 68\ mm^3$). Even more, a 100% cure rate was achieved when laser irradiation took place 3 h after injection of photosensitizer.[88] In addition, ICG, an FDA-approved cyanine dye, had been recently investigated as a potential photosensitizer by many research groups.[89–91] ICG displays strong absorption at 780 nm and intense emission at about 810 nm, providing an appropriate penetration depth in tumor tissue for PDT.

Utilization of photosensitizers-absorbing light at longer wavelengths has become an important focus of research. Successful applications of these photosensitizers in treating various tumors in deeper tissue have been demonstrated remarkable progresses for PDT.[92,93] Apart from deep penetration, an ideal photosensitizer also needs a sufficient quantum yield of triplet formation, which could induce the generation of singlet oxygen effectively. As reported, extinction of IR-700 is $2.1 \times 10^5\ M^{-1}\ cm^{-1}$ at λ_{max} of 689 nm, about 10-fold higher than that of m-THPC (Foscan; $2.2 \times 10^4\ M^{-1}\ cm^{-1}$ at 652 nm). High extinction coefficient generally means outstanding singlet oxygen generation efficiency and subsequent sufficient phototoxicity to tumor cells. Developing those photosensitizers both with longer λ_{max} and high quantum yield of triplet formation will greatly promote the practice of PDT in tumor treatment.

Accumulation of Photosensitizers in Targeted Tumor Tissue/Cell

Another drawback of photosensitizes that are generally used in clinics is that most of them exhibit limited selectivity toward tumor tissue, thus resulting in nonspecific photodamage to normal tissues. To overcome these limitations, there have been considerable efforts to enhance tumor specificity of photosensitizers. There are several approaches to improve the targeting of PDT agents, including targeting molecules conjugation and nanoparticle-mediated targeted delivery. The targeting molecules included protein, such as antibodies,[27,94] and small targeting molecules such as RGD.[95,96] Antibody-mediated PDT is a mature technique that improves photosensitizer-specific delivery. The antibodies deliver the photosensitizer to tumor cells with specific receptors overexpressed on the surface. For instance, mAb-IR-700 (conjugation between IR-700, a NIR phthalocyanine dye and mAbs) targeted tumor specifically and produced enough phototoxicity after irradiation effectively.[87] Their selective accumulation in targeted tumor tissue can locate the tumor area precisely and provides possibility for effective PDT process. However, there is a contradiction upon antibody-based PDT. To maintain targeting ability, antibodies must have a low photosensitizer-to-antibody conjugation ratio. Low conjugation ratio means that the PDT process demands a larger amount of expensive antibodies, which is adverse for the wide application of PDT. Much attention should be paid to achieve a balance between targeting affinity and conjugation ratio during designing targeted photosensitizers.

Apart from antibodies, other proteins, for example, transferrin, could also be applied to deliver photosensitizers to tumor cells via a receptor-mediated pathway.[97,98] In addition, conjugating small targeting ligands to photosensitizers is another strategy that has shown a great amount of potentials.[95,99,100] Conjugating FA to tetraphenylporphyrin could improve photosensitizers uptake in target cells *in vivo* through folate-acid-receptor-mediated endocytosis.[100] The folate acid receptors are overexpressed in many tumors and have been widely applied for target drug delivery.[101] Peptides are also skillful as small targeting ligands. Enhancement of targeting ability has been achieved through conjugation photosensitizers to various targeting peptides, including the RGD peptides and neuropilin-1-targeting peptides.[102–105] In addition, aptamer-mediated targeting strategy has been adequately demonstrated as an excellent targeting technique for fluorescence-guided PDT.[106–108] Aptamer molecules could be attached to photosensitizers via covalent conjugation[107] or noncovalent-specific affinity between aptamer and photosensitizer molecules.[108]

Nanoparticle-mediated PDT is another successful strategy for enhancing tumor tissue specificity, which could be achieved via passive, active targeting processes, or both.[109,110] Passive targeting primarily relied on the EPR effect of nanoparticles. Active targeting could be achieved through conjugating nanoparticles to biological ligands, such as antibodies, peptides, and aptamers, which show high affinity with specific surface receptors overexpressed on various tumor cells or tumor-related vascular endothelial cells.[111–115]

The specific delivery of photosensitizers is critical for the effectiveness of PTD. Because most photosensitizers used in clinics lack tumor specificity, considerable photodamage takes place in normal tissues during PDT. Compared with nontargeted photosensitizers, more ligands- or nanoparticles-based targeted photosensitizers will bind to or be taken up by tumor cells or tissues and then produce more singlet oxygen upon irradiation. Because of short half-life ($<0.04\,\mu s$) and small radius ($<0.02\,\mu m$) of the action of singlet oxygen, only biomarcomolecules proximal to areas of singlet oxygen production will be directly destroyed by PDT.[116,117] Therefore, delivering sufficient photosensitizers would be needed to produce enough phototoxicity to tumor tissues. Accordingly, targeted photosensitizers will improve distribution and reduce adverse damage to normal tissues. Thus, targeted photosensitizers largely improve effectiveness of PDT and obviously reduce systemic side effects. However, to date, very few of these targeted photosensitizers are applied in clinical practice. Handicaps mainly lie on the high cost of active targeting ligands, such as antibodies and aptamers, and the long-term stability and toxicity of the targeted photosensitizers. In addition, the complicated process of conjugation may be another potential obstacle. Apart from these common drawbacks of targeted ligands and nanoparticles, the extent of phototoxicity depends on many other factors such as light exposure dose, oxygen availability, and the time between the administration of photosensitizers and light exposure. All these factors need to be studied carefully before clinical transition.

Activatable Photosensitizers

Although advanced vehicles and fluorescence-guided laser irradiation provide enhanced tumor selectivity, there still are some side effects limiting the wide application of PDT. Some targeted photosensitizer molecules still distributed into normal tissues, especially skin tissue, resulting in unwanted photodamages. These photosensitizers distributed in skin under the sunlight will produce singlet oxygen and subsequently induce skin photosensitivity, which may result in serious sunburn or pain for up to several months after PDT.[118] Therefore, an improved strategy is creating activatable photosensitizers. In their inactive state, activatable photosensitizers remain in a quenched conformation and generate no or only a small amount of singlet oxygen upon irradiation. And it means that, even under sunshine, they will not produce singlet oxygen and induce skin photodamage obviously before specific activation. After activation by the target tissues or cells, activatable photosensitizers generate greater singlet oxygen.

Commonly two types of quenching were conducted to prepare activatable photosensitizers, which consist of fluorescence resonance energy transfer (FRET) quenching strategy[119] and "dark" nanoparticle-based quenching strategy (Fig. 7).[120–122] FRET is a dynamic quenching mechanism in which energy from the excited donor dyes is transferred to the small molecular acceptor molecules without absorption or emission of light. Apart from small molecular quenchers, "dark" nanoparticles, such as gold nanorod (GNR) and grapheme oxide, absorbing light in NIR region effectively, could also be selected as quenchers for preparing activatable photosensitizers. FRET quenching mainly consists of self-quenching and quencher quenching. Self-quenching activatable photosensitizers comprise two or more fluorophores, wherein energy from one fluorophore is transferred to another when these molecules are in close proximity.[123] Generally, these fluorophore molecules could be bonded together via short peptide linkers[119] or polymeric macromolecules.[123,124] Two fluorophore molecules linked by short peptide linker always showed both low fluorescent quenching efficiency and dissatisfied singlet oxygen quenching efficiency.[119] A preferable solution for this limitation is to conjugate more fluorophore molecules to polymeric macromolecules or nanoparticles to enhance the quenching efficiency of singlet oxygen.[125] The high quenching efficiency meant that activatable photosensitizers would not induce singlet oxygen generation when exposed to sunlight. For instance, chlorin e6 (Ce6) conjugated polylysine macromolecules (Fig. 8a) were established for improved PDT.[123] The optimized conjugates containing 15.0 ± 1.2 Ce6 molecules per polymeric chain exhibited only about 15% fluorescence and 12% singlet oxygen, respectively, compared with free Ce6 at equal molar concentration. The polylysine backbone was sensitive to tumor-associated proteases, and could be cleaved especially by cathepsin B, highly expressed in many tumor cells.[124,126] After cleavage, the detached photosensitizers became highly fluorescent and generated singlet oxygen greatly because there was no more FRET occurring between the adjacent photosensitizers (Fig. 8b). Macromolecules-based activatable photosensitizers improved singlet oxygen quenching efficiency compared with two fluorophore molecular conjugates. However, it is difficult to achieve more excellent activatable photosensitizers with higher fluorescence or singlet oxygen quenching efficiency via this method. Conjugating more Ce6 molecules to macromolecular chain could quench more than 95% fluorescence and singlet oxygen generation, but too many Ce6 decreased active sites for enzymatic cleavage. Even after protease treatment for up to 4 h, most of the conjugates were still quenched (Fig. 8c). It was important to conjugate appropriate photosensitizer molecules to a polymer chain to achieve a balance between quenching efficiency and sufficient enzymatic cleavage sites.

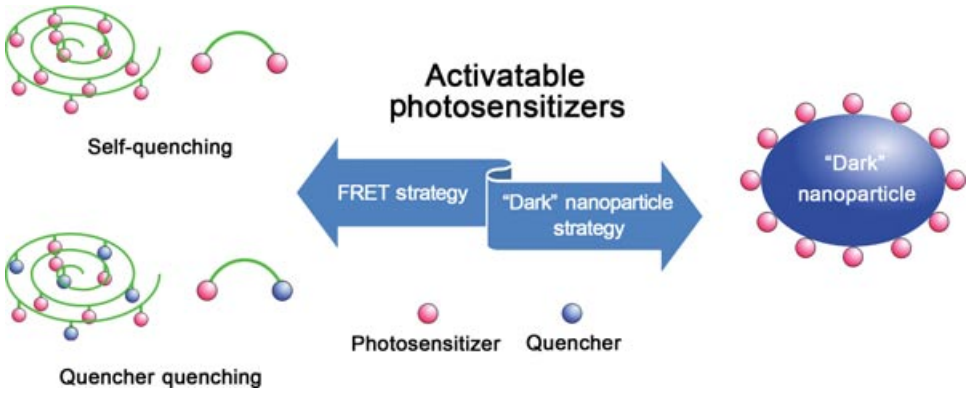


Figure 7. Schematic illustration of quenching modality of activatable photosensitizers. It mainly includes FRET quenching strategy and "dark" nanoparticle-based quenching strategy. FRET quenching mainly consists of self-quenching and quencher quenching. In which, fluorophores themselves or dark quenchers are used for quenching processes. Additionally, "dark" nanoparticle, which can absorb NIR light, are also designed as quenchers.

To decrease the background fluorescence production of and singlet oxygen in inactive state, an alternative strategy was conjugating dark quencher to fluorophore molecules.[119] A dark quencher is substance-absorbing excitation energy from a fluorophore when the two are close together. Subsequently, the fluorophore's emission will be suppressed. Different fluorophore molecules need different dark quenchers. Greater spectral overlap between fluorescent emission of fluorophore molecules and absorption spectrum of dark quenchers induces effective quenching of photosensitizer. For instance, black hole quencher 3 (BHQ3), a dark quencher, covalently attached to pyropheophorbide-α could quench 95% of the fluorescence emission and 93% of the singlet oxygen production.[119] Many other quencher–fluorophore conjugates were synthesized and used as successful activatable photosensitizers in PDT.[127,128]

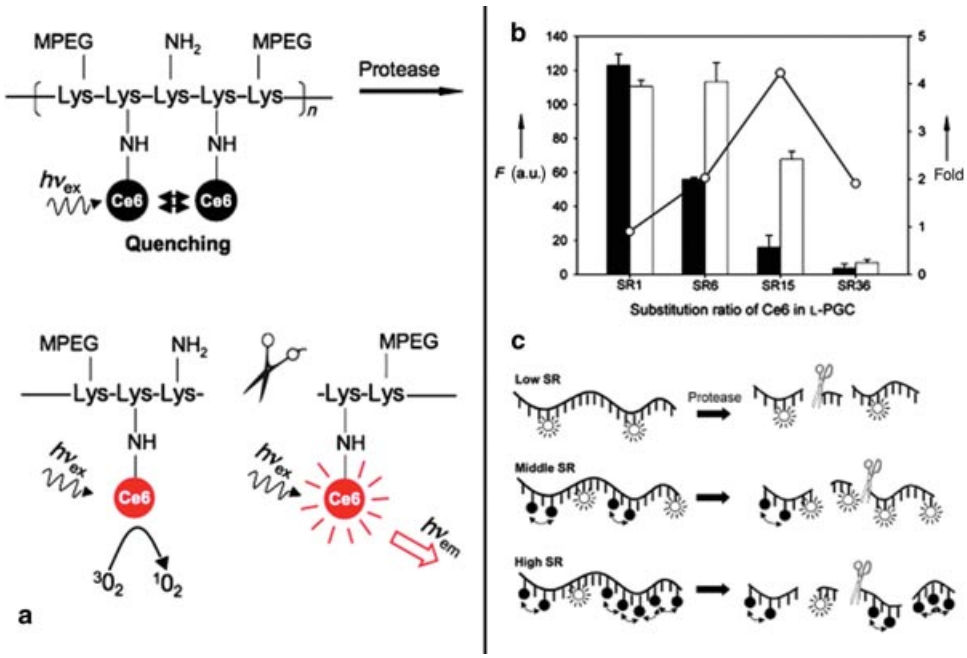


Figure 8. Schematic illustration of macromolecule-based activatable photosensitizer. Both NIR fluorescence and singlet oxygen generation are markedly decreased by conjugating multiple Ce6 molecules to a polypeptide chain. Protease-mediated cleavage of the chain results in detached, phototoxic, and fluorescent Ce6 molecules (a and b). Ce6-molecules-dependant photosensitizer activation after enzyme treatment. With the increasing of Ce6 molecules in a polypeptide chain, the number of free lysine residues decreases, resulting in fewer sites for enzymatic cleavage (c).[123]

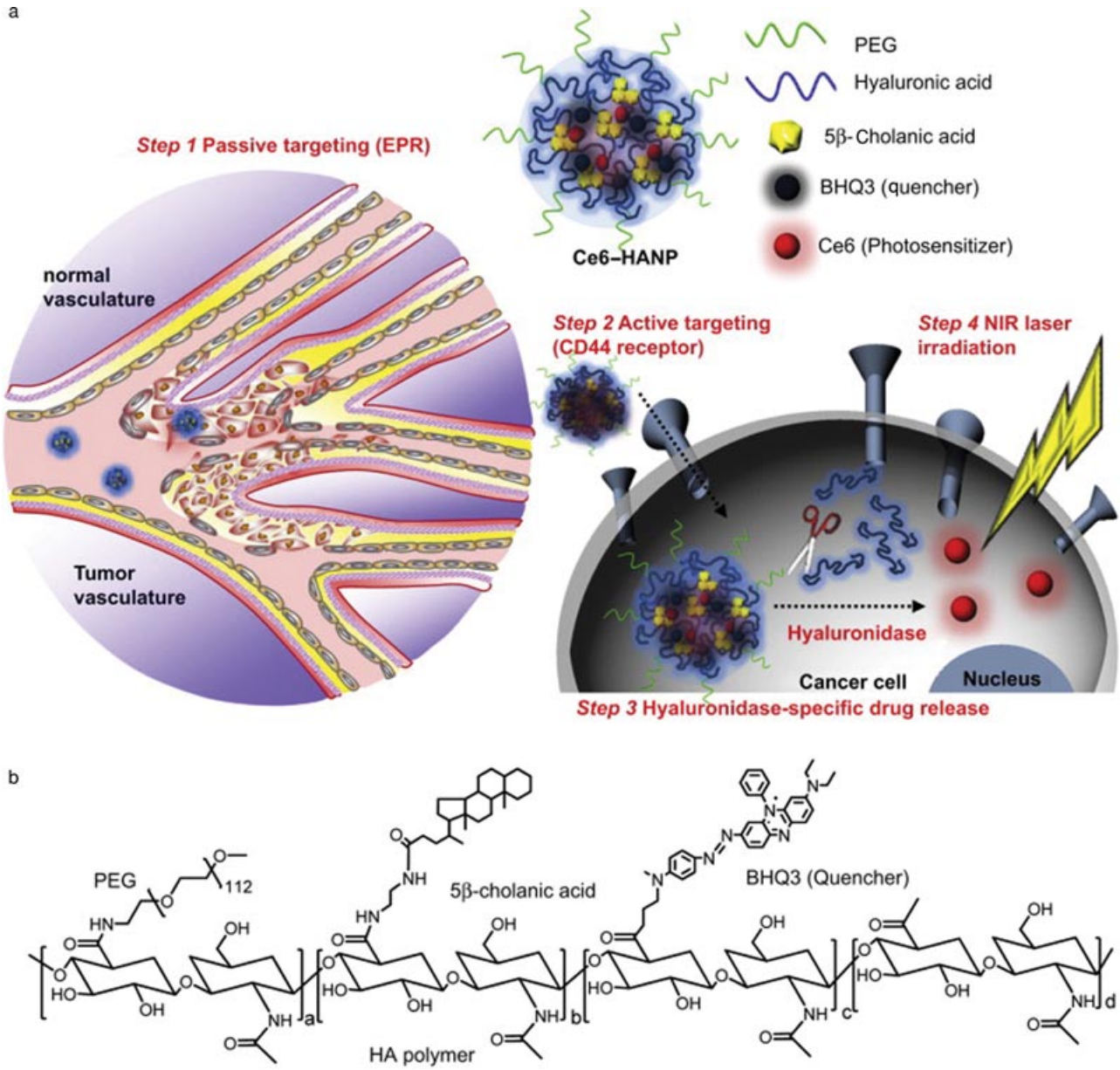


Figure 9. Schematic illustration of tumor-targeting hyaluronic acid nanoparticle containing Ce6 (Ce6–HANP) (a). Chemical structure of HANP. BHQ3 acted as quencher and 5β-cholanic acid formed hydrophobic core of nanoparticles (b).[129]

Small molecule photosensitizers exhibited systemic biodistribution and commonly lack of tumor specificity. Then, tumor-targeted nanoparticles containing quenchers and fluorophores were developed as a new modality, which showed improved tumor accumulation. For instance, tumor-targeting hyaluronic acid (HA) nanoparticles (Ce6–HANPs; Fig. 9a) contained both BHQ3 (quencher; Fig. 9b) and Ce6 (fluorophore).[129] Because of effective quenching activity of BHQ-3, the fluorescence intensity and singlet oxygen production of Ce6–HANP was much lower than that of free Ce6 before activation. Furthermore, Ce6–HANPs accumulated in tumor tissue showing 2.4-fold higher than that of free Ce6 because of both EPR effect and high affinity between HA molecules and CD44, the HA receptor on the surface of tumor cells. Once taken up by tumor cells, free Ce6 molecules were rapidly released from Ce6–HANP because the backbone of HA molecules were cleaved by hyaluronidases, which were abundant in tumor cells. And then the released Ce6 molecules were dequenched and generated singlet oxygen effectively upon laser irradiation.

"Dark" nanoparticles, which absorb NIR light effectively, have also been selected as ideal quenchers for preparing activatable photosensitizers. Similar

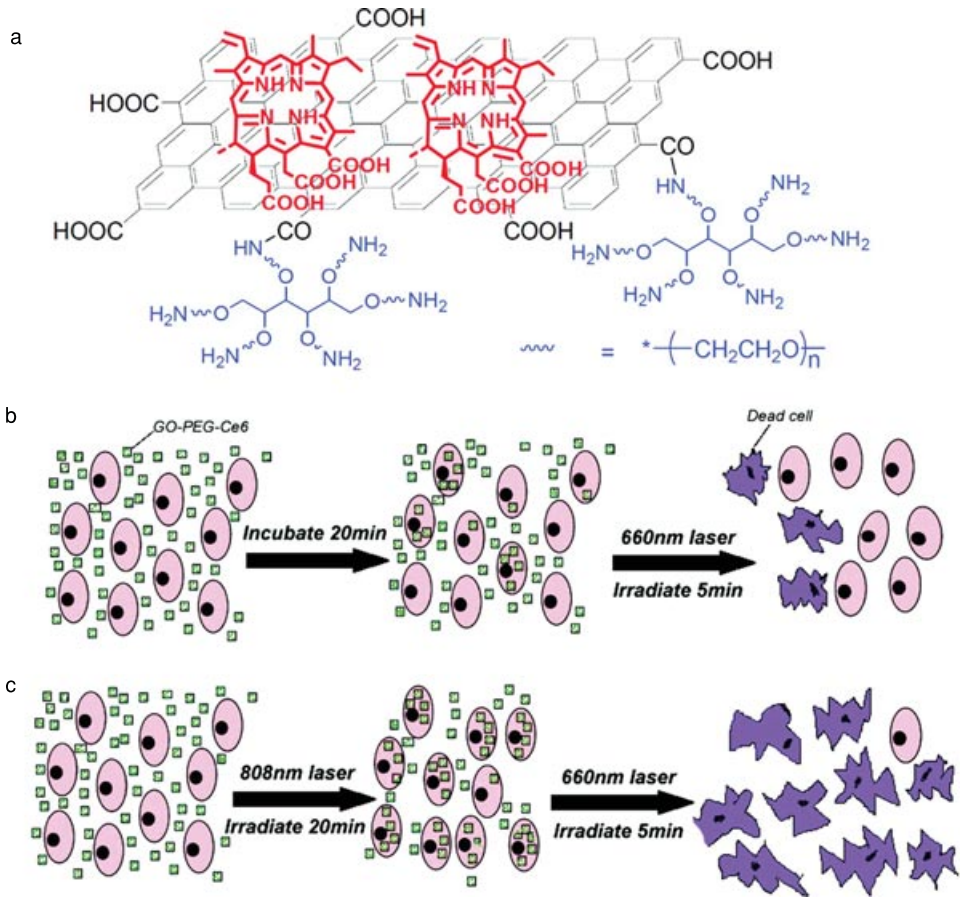


Figure 10. Schematic illustration of GO–PEG–Ce6 (a) and photothermally active photodynamic therapy (b and c). KB cells were incubated with GO–PEG–Ce6 in the dark for 20 minutes and then irradiated by a 660 nm laser (b). To confirm the photothermal effect, tumor cells incubated with GO–PEG–Ce6 were first exposed to an 808 nm laser (PTT) before PDT treatment. PTT itself almost does not influence the cell viability, but it could active quenched Ce6 molecules. After activation by PTT, phototoxicity of GO–PEG–Ce6 was improved (c).[122]

with small dark quencher molecules, fluorophores will be quenched effectively when they are in close to the surface of "dark" nanoparticles. When the fluorophores are activated and move away from the surface of these nanoparticles, laser irradiation will result in singlet oxygen generation greatly. Furthermore, these nanoparticles with immense absorption coefficient in NIR region can also be used as efficient photothermal agents. Therefore, these nanoparticles combining PTT and activatable PDT attract more attentions recently. Tian et al.[122] reported a photothermally enhanced PDT agent consisting of branched PEG-functionalized nanographene oxide and Ce6 (GO–PEG–Ce6; Fig. 10a). Because of the close distance between Ce6 and nanographene oxide, 80%–90% of Ce6 fluorescence was quenched. Meanwhile, 50%–60% of the singlet oxygen generation of GO–PEG–Ce6 was quenched, too. After endocytosis, PTT was conducted and the temperature within tumor tissue rises. Owing to the increased temperature, Ce6 molecules breaked the hydrophobic interactions and slowly released from the surface nanographene oxide. It resulted in singlet oxygen generation upon laser irradiation (Fig. 10b). Another nanoparticle with high NIR region absorption, GNR, was selected to enhance singlet oxygen quenching and decrease nonspecific generation of singlet oxygen in normal tissues exposed to sunlight. A nanocomplex formed with GNR and photosensitizer AlPcS4 (GNR–AlPcS4; Fig. 11a) was conducted as a multifunctional nanoplatform for *in vivo* imaging and therapy.[121] Surprisingly, GNR–AlPcS4 exhibited only 0.4% of fluorescence intensity and 0.03% of singlet oxygen generation compared with free AlPcS4. It showed great superiority in low nonspecific activation of singlet oxygen generation. After accumulated in tumor tissue or cells, NIR laser was used to conduct PTT and simultaneously the generated heat in tumor tissue could induce quenched AlPcS4 molecules detaching from the surface of GNR. Then, AlPcS4 molecules were activated and produce singlet oxygen greatly (Figs. 11b and 11c). For simultaneously enhanced active tumor targeting, an

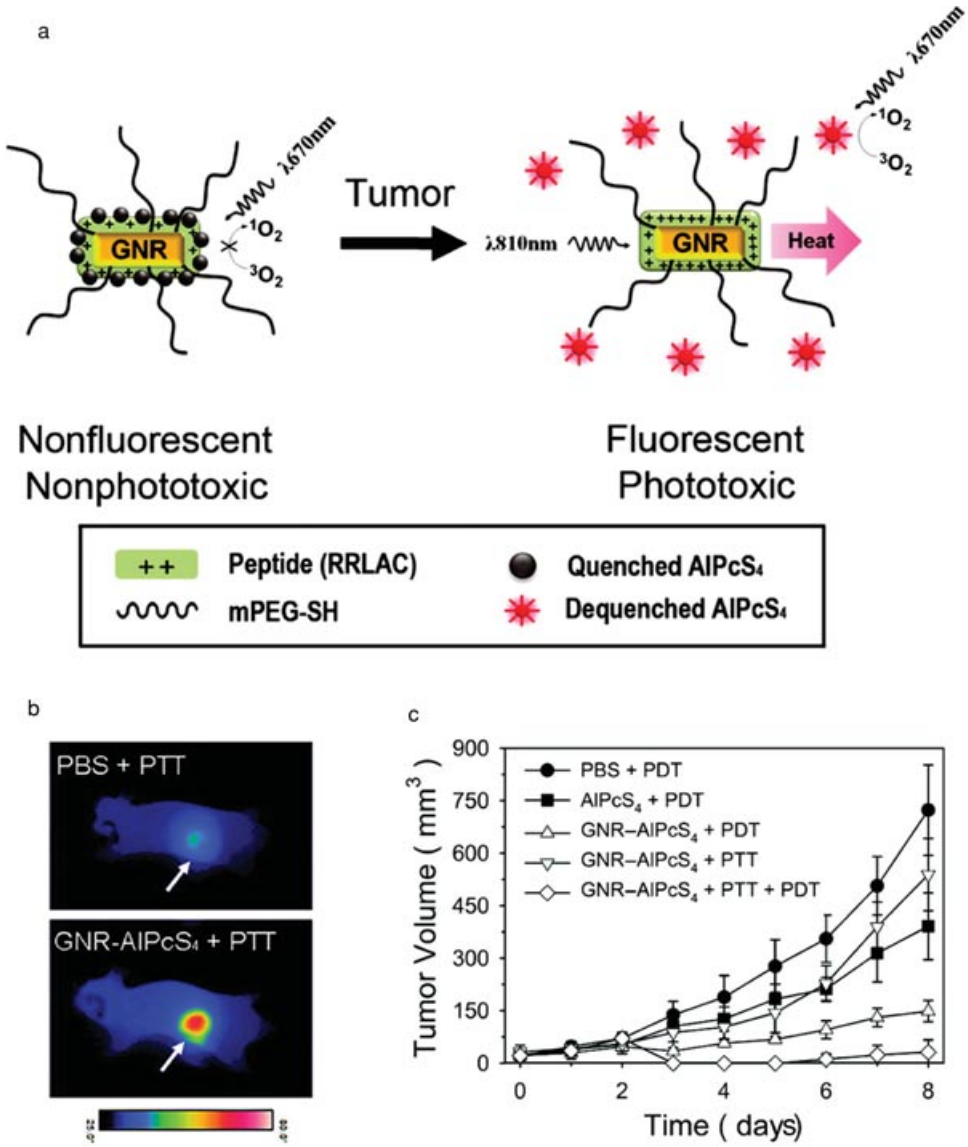


Figure 11. Schematic diagram of the GNR–AlPcS4 complex for NIR fluorescence imaging and tumor phototherapy (a). Thermographies captured after 1 min of light irradiation in the tumors of GNR–AlPcS4 and PBS-administrated mice (b). Tumor size in different therapy groups (c).[121]

aptamer switch probe linking Ce6 to the surface of GNR (AuNR–ASP–Ce6) was designed and synthesized.[120] While binding with receptors on target tumor cells, conformation of aptamer switch probe changed, driving Ce6 molecules away from the surface of GNR, thereby dequenching the activatable photosensitizers and producing singlet oxygen for PDT effectively (Fig. 12a). As a result, the CCRF–CEM (Human acute lymphocytic leukemia (ALL) cell line) tumor cell viability decreased to about 80% ($p < 0.05$) upon light irradiation. In contrary, phototoxicity of AuNR–ASP–Ce6 to Ramos cells was negligible, with cell viability of above 95%. This may be due to Ramos cells having no aptamer receptors on their surface (Figs. 12b and 12c).

All these activatable photosensitizers had been demonstrated to improve PDT effectively. Activatable photosensitizers could potently and specifically induce diseased cell death according to their different microenvironment, such as specific enzyme expression. Recently, several thermal-sensitive activatable photosensitizers have also been designed and demonstrated as promising platforms for PDT. Moreover, many other specific sensitive factors such as low pH and reductive condition in specific subcellular organelles could also be applied as triggers for activatable photosensitizers. In consideration of small radius of the action of singlet oxygen, delivering more activatable photosensitizers into tumor cells by conjugating targeted ligands will also be a promising strategy. Overall, development of advanced activatable photosensitizers will be a significant orientation for treating tumors in the coming years.

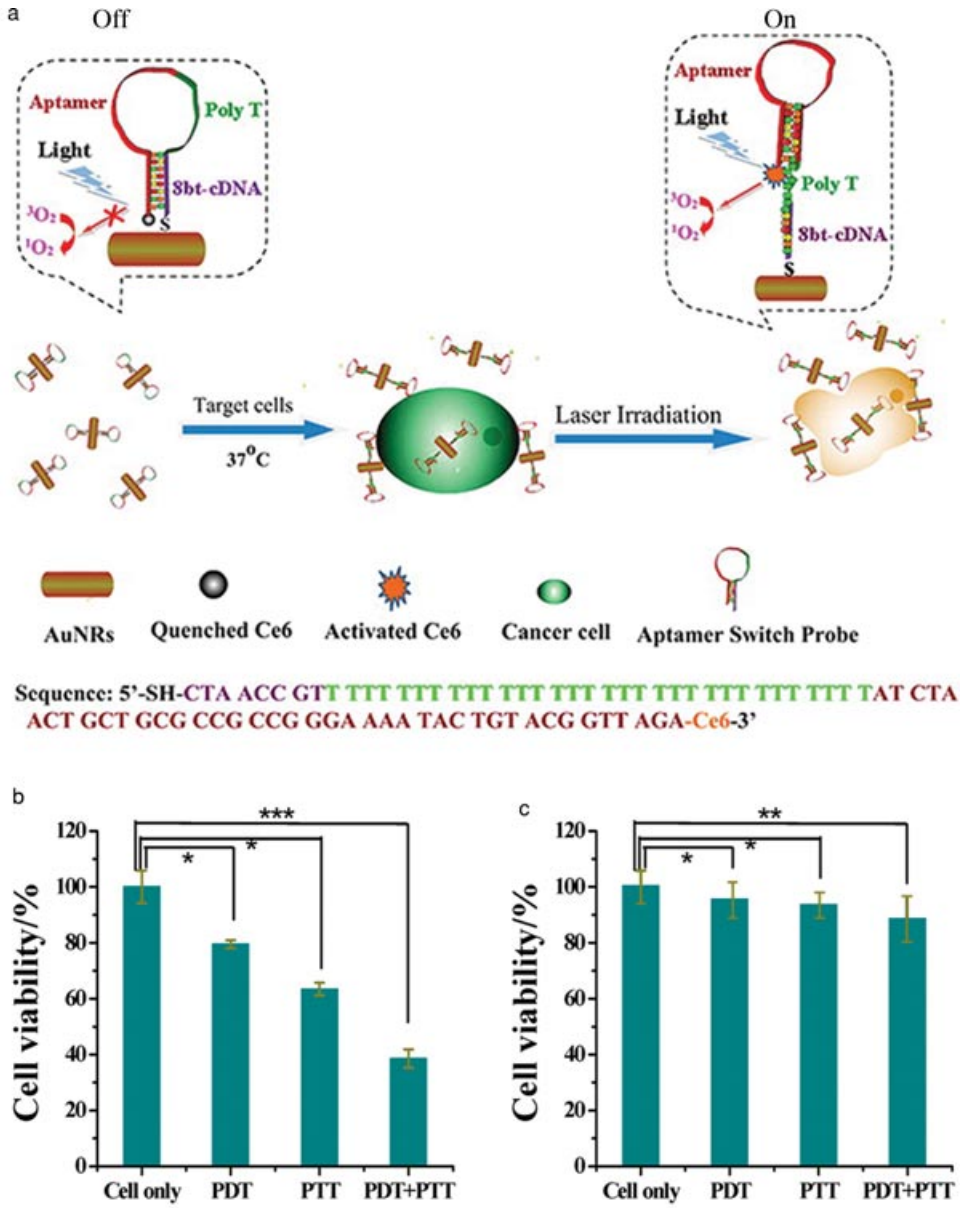


Figure 12. Schematic diagram of an aptamer switch probe AuNR–ASP–Ce6 (a). With white light irradiation (PDT), the CCRF–CEM tumor cell viability decreased to about 80% ($p < 0.05$). However, phototoxicity of AuNR–ASP–Ce6 to nontarget Ramos cells was negligible, with cell viability of above 95% (b and c).[120]

CONCLUSIONS

The application of NIR dyes in tumor researches has obtained rapid and outstanding progress. For tumor detecting *in vivo*, NIR dyes have low autofluorescence and deep penetration in NIR region, which greatly improve background interference and bring detectable fluorescent signal from deep-seated tumors. To date, some NIR dyes, such as 5-ALA, have been applied in clinics for detecting tumor margins or microcancer lesions before operation. Additionally, other NIR dyes, for instance, ICG and bevacizumab–IRDye800CW conjugate, have completed preclinical studies and are currently undergoing clinical trial for early cancer diagnosis. To detect tumor precisely, improving tumor signal and decreasing background signal are two main promising strategies. Enhancing tumor signal could be achieved by improving tumor accumulation of NIR dyes, such as developing novel NIR dyes with acceptable solubility and sufficient tumor specificity, and conjugating NIR dyes with targeted vehicles. Moreover, developing various activatable imaging agents that could be activated by low pH, reductive condition, and specific enzymes in tumor cells has been conducted to decrease background signal effectively.

Meanwhile, as photothermal effect, heat released from excited NIR dyes could efficiently destroy tumor

tissue. Importantly, visual imaging could efficiently help to guide specific therapy and decrease side effect. Recently, many targeted free NIR dyes have been reported. Whether these targeted free NIR dyes can be applied as theranostic agents in PTT really deserves further research. And NIR PDT is another emerging strategy for tumor inhibition. PDT shows great advantages in its noninvasiveness and repeatability compared with surgery and radiation. Recently, many photosensitizers, such as BPD-MA and m-THPC, have been applied widely and successfully in clinics. And many other advanced photosensitizers, such as 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH), are tested in clinics for application in PDT. Like PTT, PDT also brings dual specificity to the therapeutic procedure because only the imaged tumor area will be irradiated with laser while the surrounding normal tissue is minimally hurt. All these demonstrate that NIR dyes have great potential in tumor clinical application, either diagnosis or therapeutics. However, tumor specificity, safety, and molecular stability are critical for transition from bench to bedside. Ideal tumor detecting agents need high T/B ratio, which ask for both tumor specificity and low background signal. Most of the widely studied NIR dyes exhibit poor stability and lack of tumor selectivity. For PTT, the low photostability and subsequently low efficiency of heat generation adversely affect final treatment efficacy of NIR dyes. Because of photodamage in normal tissues, undesired distribution also limits wide application of PDT and PTT. Therefore, different strategies have been developed to overcome these limitations. Targeting ligands can be conjugated to NIR dyes directly or NIR-dyes-loaded nanoparticles to achieve enhanced specific accumulation in tumor tissues or cells, which greatly improve molecular stability and decrease its self-toxicity. More interestingly, the activatable modality provides a promising way for optimizing NIR dyes. The controlled and specific molecular activation would greatly decrease the background fluorescence and undesired damage.

As expected in this manuscript, an ideal NIR dye acting as an imaging and therapeutic agent (1) is soluble in relevant buffers, human blood, or body fluids; (2) is sufficiently stable during transportation and storage; (3) is easy to be synthetic or extractive; (4) is extremely low toxic; and (5) is sufficiently tumor specific. There being considerable challenges; the development of NIR dyes for tumor diagnosis and therapeutics still represents an exciting and interesting research area.

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