Current trends and key considerations in the clinical translation of targeted fluorescent probes for intraoperative navigation

Renfa Liu# | Yunxue Xu# | Kun Xu | Zhifei Dai

INTRODUCTION

Cancer is one of the major causes of human death. The most common treatment for many localized tumors is surgery in combination with chemotherapy and/or radiotherapy.[1] The rapid development of fluorescence imaging for intraoperative navigation has spurred further development of targeted fluorescent probes in the past decade. Only a few nontargeted dyes, including indocyanine green and methylene blue, are currently applied for fluorescence guided surgery in the clinic. While no targeted fluorescent probes have been approved for the clinic, a number of them have entered clinical trials. These probes have emission wavelengths in the visible and near infrared (NIR)-I (700-900 nm) range. Among them, activatable probes and nanoprobes have generated special interest. Compared with NIR-I fluorescent probes, NIR-II (1000-1700 nm) fluorescent probes exhibit better intravital performance in terms of increased penetration depths, reduced tissue autofluorescence, and higher signal-to-background ratios. However, more challenges are expected before the successful translation of NIR-II probes from bench to bedside. This review provides a brief overview of targeted fluorescent probes under clinical evaluation and recent achievements in the field of NIR-II fluorescence imaging. In addition, we outline key considerations concerning the design of fluorescent probes for clinical translation.

Abstract

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KEYWORDS

clinical translation, fluorescence imaging, intraoperative navigation, targeted fluorescent probes, tumor surgery

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REVIEW

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| Name | Fluorophore | Spectra (excitation/emission) | Target | Route of administration | Cancer type | Clinical status |
|------|-------------|-------------------------------|--------|-------------------------|-------------|----------------|
| **Fluorescent probes in the visible wavelengths** | | | | | | |
| EC-17 [136] | FITC | 471/519 nm | Folate receptor | Intravenous | Lung cancer and breast cancer | Phase 1 |
| ASY*-FITC [17] | FITC | 471/519 nm | Cyclophilin A | Topical | Ovarian cancer and renal cell carcinoma | Phase 2 |
| Colon KCC [18] | FITC | 471/519 nm | Unknown | Topical | Esophageal cancer | Phase 1 |
| PARPi-FL [21] | BODIPY FL | 507/525 nm | PARP1 | Topical | Colonic cancer | Phase 1 |

| **NIR-I fluorescent probes in the 700 nm channel** | | | | | | |
| GE-137 (EMI-137) [126] | Cy5 | 646/678 nm | c-MET | Intravenous | Colonic cancer | Phase 2 |
| QRH-882260 [138] | Cy5.5 | 671/710 nm | EGFR | Topical | Lung cancer, Barrett esophagus, and papillary thyroid cancer | Phase 1 |
| SGM-101 [139] | BM104 | 685/705 nm | CEA | Intravenous | Colon cancer and cholangiocarcinoma | Phase 1 |

| **Fluorescent lectins [140]** | | | | | | |
| Panitumumab-IRDye800CW [16] | S0456 | 776/793 nm | Folate receptor | Intravenous | Ovarian cancer and lung cancer | Phase 3 |
| KSP-910638Gi [18] | S0456 | 772/788 nm | Integrin | Intravenous | Oral cancer | Phase 2 |
| KSP-QRH-E3-IRDye800 [141] | S0456 | 756/776 nm | eHsp90 | Intravenous | Breast cancer | Phase 1 |

(Continues)
significant advantages of novel fluorophores that emit in the NIR II region (900-1700 nm), such as carbon nanotubes, metal chalcogenide quantum dots (QDs), rare-earth (RE)-doped nanoparticles, and donor-acceptor-donor (D-A-D) dyes. This review will first summarize the molecular designs of the targeted fluorescent probes under clinical evaluation followed by recent advances in the chemical design of emerging NIR-II fluorophores. Finally, key issues related to the clinical translation of targeted fluorescent probes will be discussed.

**CURRENT TRENDS IN THE DEVELOPMENT OF FLUORESCENT PROBES FOR CLINICAL TRANSFORMATION**

**Fluorescent probes in visible wavelengths in clinical studies**

The initial report of the use of fluorescent probes for surgical navigation dates back to 1947, when sodium fluorescein was used to discriminate brain tumors (Figure 1A). At 3-8 h
post injection, superficial brain tumors were observed with a vivid yellow color under the irradiation of UV light. The increased fluorescence of brain tumors was a result of passive accumulation of sodium fluorescein due to the impaired blood-brain-barrier in malignant brain tumors. Another fluorescent probe that has been approved for use in the clinic is 5-ALA (Figure 1B), which is a naturally occurring precursor for heme synthesis in mammalian cells. Following oral administration, 5-ALA preferentially accumulates in tumor and can be transformed into endogenous fluorescent molecule, protoporphyrin IX (PPIX) (Figure 1C). Compared with conventional microsurgery with white light, 5-ALA-assisted surgery resection allows for more complete glioma resection and increases 6-month progression-free survival rate by two-fold.\cite{11} However, 5-ALA has some shortcomings, such as phototoxicity to normal tissues and the inability to clearly discriminate the tumor boundary.\cite{12}

Inspired by these initial success, additional fluorescent probes with specific targets have been developed in the past decade for fluorescence imaging-guided surgery of several tumor types. Folate receptor \( \alpha \) (FR\( \alpha \)) is a promising target because it is highly expressed in various epithelial cancers, including 90-95\% of epithelial ovarian cancers (a common cancer with a high mortality rate), while its expression remains at a low level in normal cells.\cite{13} This phenomenon motivated researchers to conduct studies on FR\( \alpha \)-targeting fluorescent probes for tumor-specific fluorescence imaging.\cite{14} For the first time, in 2011, EC-17, a folic acid-bearing fluorescein isothiocyanate (FITC) (Figure 1D), was developed for intraoperative tumor-specific fluorescence imaging.
imaging in patients with ovarian cancer. In three out of four patients with malignant tumors, tumor-specific fluorescence was observed intraoperatively. However, mild adverse effects were detected after the administration of EC-17 at a dose of 0.3 mg/kg. In a further study with a larger patient cohort and reduced dose (0.1 mg/kg), EC-17 helps to successfully detect intraoperative fluorescence of 57 lesions among 12 patients, 44 of which were confirmed to be FRα positive by histopathology. Seven of these lesions were not detected by the naked eye or palpation. These results demonstrated the feasibility of FRα-targeted fluorescence imaging for the intraoperative detection of FRα-positive tumor lesions. Finding a suitable tumor-targeting ligand is a prerequisite for fabricating a suitable targeted fluorescent probe. Wang et al. used phage display technology to screen peptides for tumor targeting. The ASNYDA peptide was identified to have a higher binding affinity to the plasma membrane of Barrett’s neoplastic cells than nonneoplastic cells. This peptide was conjugated with FITC through a GGGSK linker to prevent steric hindrance, resulting in the fluorescent probe ASY*-FITC (Figure 1E). In first-in-human studies, the fluorescence intensity of esophageal neoplastic tissues was ~3.8-fold higher than that of Barrett’s esophagus and the squamous epithelium following a topical spray treatment of ASY*-FITC onto the esophageal tissues of interest. Using a similar strategy, an FITC-labeled KCCFPAQ peptide (named Colon KCC) was developed for the detection of colonic dysplasia.

In addition to receptors on the cell membrane, targeted fluorescent probes can also be developed for intracellular targets. The DNA repair enzyme poly(ADP-ribose) polymerase 1 (PARP1) plays a key role in the maintenance of genomic stability and is highly expressed in rapidly growing tumors. Inhibition of PARP1 causes multiple double strand breaks, and in tumors with BRCA1, BRCA2 or PALB2 mutations, these breaks cannot be efficiently repaired, leading to the death of the tumor cells. Therefore, there has been an intense clinical focus on the inhibition of PARP1 for the treatment of various cancers. Reiner et al. fabricated the small-molecule PARP1-targeting fluorescent probe PARPi-FL by conjugating a boron-dipyrromethene fluorescent dye (BOD-IPY FL) with the scaffold of olaparib, an FDA-approved PARP1 inhibitor (Figure 1E). PARPi-FL and olaparib have a similar binding affinities to PARP1, and the hydrophobic nature of PARPi-FL makes it a cell-permeable probe that can be cleared rapidly if it is not bound to the target. In preclinical animal models and fresh human biospecimens, PARPi-FL has been able to detect oral, oropharyngeal, and esophageal epithelial cancers after intravenous or topical administration. In a clinical trial, PARPi-FL demonstrated the feasible discrimination of malignant oral cancer lesions after administration of a topically applied mouthwash. A mouthwash with a 500 nM PARPi-FL solution for 1 min allows discriminating the oral cancer from surgical resection margin with more than 95% sensitivity and specificity (Figure 1G and H).

**NIR-I fluorescent probes under clinical evaluation**

Despite the many clinical benefits obtained with visible wavelength fluorescent probes, their application in humans has been largely compromised by the poor tissue penetration of visible light and significant interference from tissue autofluorescence. In contrast, fluorescence imaging in the NIR-I window (700-900 nm) can afford deeper tissue imaging with a significantly increased SBR due to reduced photon scattering and absorption by biological tissues along with lower tissue autofluorescence. Therefore, an increasing number of preclinical and clinical studies have focused on the development of targeted fluorescent probes in the NIR-I window. Currently, MB and ICG are the only two NIR-I fluorescent probes approved in the clinic with emission peaks at approximately 700 and 800 nm, respectively (Figures 2A and 3A). They have been used in fluorescence imaging-guided surgery for SLN mapping, the detection of vital structures (eg, bile ducts and ureters), assessment of tissue perfusion, and imaging-guided resection of certain tumors. However, both are nonspecific fluorescent probes that do not bind to tumor cells. Therefore, a number of tumor-targeting NIR-I fluorescent probes have been developed in the past few years for surgical navigation with an improved SBR. Almost all of the targeted NIR-I fluorescent probes under clinical evaluation use cyanine dyes, which consist of two aromatic nitrogen-containing heterocyclic terminal groups linked by a polymethine bridge. Cyanine-based NIR fluorescent probes can generally be divided into two types based on the length of the polymethine chain. One type is the pentamethine cyanine fluorophore with an emission peak wavelength at ~700 nm, similar to MB. The other type is the heptamethine cyanine fluorophore emitting at ~800 nm, similar to ICG.

**NIR-I fluorescent probes in the 700 nm channel**

SGM-101 is an antibody-dye conjugate in which the fluorophore BM-104 is conjugated with a chimeric monoclonal antibody SGM-ch511 against carcinoembryonic antigen (CEA) (Figure 2B). CEA is highly expressed in several tumors (including colorectal, pancreatic, and lung cancers), and CEA expression in tumor tissues is on average 60 times higher than that in healthy tissues. BM-104 is a derivative of the dye Cy5.5, with peak absorption and emission at ~700 nm. BM-104 is surrounded by four sulfonic acid groups and has a carboxylic acid group, which can be functionalized with N-hydroxysuccinimide (NHS) reactive group to react with free amino groups on the antibody surface. The antibody-dye conjugate can be made by mixing the SGM-ch511 antibody with NHS-activated BM-104 at a certain ratio. By varying the initial feeding ratio, the optimal conjugate with an average final dye/antibody ratio of 1.6 was selected for its relatively high specific binding to CEA and low nonspecific binding. Since there are several free amino groups on the antibody surface, BM-104 randomly conjugates with the antibody. At the optimal ratio, 31%, 21%, 46%, and 2% of the antibodies conjugated with 0, 1, 2, and 4 BM-104 dye molecules, respectively. Due to the relatively longer half-life of the intact antibody compared with antibody fragments, the intact antibody-dye conjugate allows a longer period of time for intraoperative imaging after injection. In a dose-escalation pilot study in patients with colorectal cancer, the highest SBR (mean SBR 6.10±0.42) was obtained 4 days after the administration of SGM-101 at a dose of 10 mg.
In addition to various membrane-associated proteins, the cell surface is also coated with a variety of glycans that play an important role in intercellular communication. Glycan expression has been shown to be altered in various cancer types, including colorectal, esophageal, and pancreatic cancers. Lectins are a family of glycan-specific proteins that are constituents of the normal human diet, stable, and inexpensive to produce. With lectin array technology, wheat germ agglutinin (WGA) was selected as the candidate lectin to detect changes in glycan expression on the epithelial cell surface. Topically administered fluorescently labeled WGA can help to detect esophageal dysplastic lesions, which are undetectable using a conventional endoscopy, with a high SBR of over 5 (Figure 2C). Unlike other targeted fluorescent probes, the fluorescence signal on the luminal surface epithelium is reduced in dysplastic lesions. After topically spraying the esophagi with fluorescently labeled WGA, the esophagus was imaged with a clinical fluorescence-capable endoscope and the dysplastic lesions could be detected with low fluorescence. The fluorescently labeled lectins were also demonstrated to be feasible for the detection of colorectal dysplasia.

Compared with high-molecular-weight proteins, small-molecular-weight short peptides are advantageous due to their lower immunogenicity, increased permeability, and ease of manufacture as a single compound with a definite structure. GE-137 is a fluorescently labeled peptide composed of a modified Cy5 dye and a water-soluble 26 amino acid cyclic peptide targeting the extracellular domain of human c-Met, which is overexpressed in a variety of carcinomas and parts of benign diseases (Figure 2D). This particular Cy5 dye was chosen from four Cy5 derivatives for its relatively high photostability, improved brightness, and reduced nonspecific binding. The cyclic peptide was screened using the M13 phage display technology on the basis of its high binding affinity for c-Met with no effect on the downstream signaling pathway. GE-137 was fabricated by conjugating the c-Met-targeting peptide with the Cy5 dye via a GGGK linker peptide. The binding affinity of GE-137 to human c-MET was determined to be as low as 3 nM ($K_d$). In the first-in-human study, GE-137 was demonstrated to be safe and feasible for the detection of colorectal polyps. GE-137 is renally cleared and the background clearance half-time was $\sim 2$ h 30 min at all doses. The optimal imaging time point was determined to be 3 h. The fluorescence imaging based on GE-137 enabled better polyps detection than traditional methods. In 15 patients with high risk of colorectal neoplasia, fluorescence imaging with GE-17 detected 17 lesions that are missed by white light inspection.
FIGURE 3  Chemical structures of the NIR fluorescent probes in the 800 nm channel. (A) ICG. (B) IRDye800CW. The free carboxylic acid group for conjugation is labeled in red. (C) KSP-QRH-E3-IRDye800. (D) 68Ga-BBN-IRDye800CW. (E) OTL-38. The unique tyrosinate moiety is labeled in red. (F) ZW800. The free carboxylic acid group for conjugation is labeled in red. (G) HS-196. (H) BLZ-100. (I) LS301
NIR-I fluorescent probes in the 800 nm channel

IRDye800CW-labeled NIR fluorescent probes

Fluorescence imaging in the 800 nm channel is advantageous over that in the 700 nm channel with further increased tissue penetration and reduced autofluorescence. Since ICG is the only clinically approved NIR dye emitting at ~800 nm, extensive research has been done to improve the optical properties of cyanine derivatives and availability for conjugation to targeting ligands with the goal of creating targeted fluorescent probes in the 800 nm channel.[7,29] IRDye800CW is one of the most widely used fluorophores, as it has a certain dominance in its structure (Figure 3B). The structures of cyanine dyes have a certain logic to follow. With extension of the polymethine chain, the wavelengths of the excitation light and emission light of the cyanine dye also increase. However, additional studies have described side effects from the polymethine chain being too long, such as prone to symmetry breaking and significant solvatochromism-caused quenching in polar solvents. So, new strategies have come out, such as IRDye800CW, to introduce a rigid cyclohexyl moiety in the middle of the polymethine chain, which can significantly improve the photostability and decrease the aggregation of cyanine dyes.[30] Moreover, sulfonic acid or carboxylic acid groups have been suggested to increase the solubility, fluorescence intensity, and Stokes shift of cyanine dyes.[31] The carboxylic acid group can be functionalized with either a maleimide or NHS reactive group, allowing its conjugation with various biomolecules.[32] Therefore, this structure makes IRDye800CW a general-purpose NIR fluorophore with the excellent qualities of safety, and good pharmacokinetics and biodistribution.

IRDye800CW-based targeted fluorescent probes were evaluated in multiple clinical trials by conjugating them to various targeting biomolecules. Some antibodies that have been approved for tumor therapy are labeled with IRDye800CW, which utilizes the free amino or thiol groups on the surface of the antibodies.[33] Several clinical trials have demonstrated the feasibility and safety of IRDye800CW-labeled antibodies for surgical guidance. For example, panitumumab-IRDye800CW and cetuximab-IRDye800CW, both of which specifically bind to the extracellular domain of epidermal growth factor receptor (EGFR), are undergoing clinical evaluation for head and neck squamous-cell carcinomas overexpressing EGFR.[34] Bevacizumab is a humanized monoclonal antibody that targets vascular endothelial growth factor (VEGF)-A, an angiogenic growth factor, which is often involved in tumor-induced angiogenesis.[35] Bevacizumab-IRDye800CW is currently under investigation in several clinical trials for fluorescence imaging-guided surgery of breast cancer and colorectal cancer.[36] The use of these FDA-approved targeting antibodies with well-established pharmacokinetic and safety data is perhaps a relatively safer and more cost-effective method for developing targeted fluorescent probes.[37]

In addition to antibodies, IRDye800CW-labeled peptides have also been evaluated in several clinical trials. Wang et al. identified the EGFR-targeting peptide QRHKPRE (QRH*) using phage display technology and the KSPNPFRF (KSP*) peptide targeting human epidermal growth factor receptor 2 (HER2) using a structural model by optimizing the amino acids to increase the likelihood of hydrophobic/hydrophilic interactions with the target.[38] These two peptides were fluorescently labeled (with Cy5.5 as QRH-882260 and IRDye800CW as KSP-910638G, respectively) for the detection of neoplastic tissues in the gastrointestinal tract after topical administration to humans. To further enhance the binding affinities of the fluorescent peptides, these two heptapeptides were combined to form a heterodimer by introducing a linker with a variable poly(ethylene glycol) (PEG) moiety ranging in size from 17 to 74 atoms. The triethylene glycol linker (E3) was found to provide optimal spacing for the heterodimer to bind to EGFR and HER2. In cellular experiments with human OE33 cells, fluorescently labeled heterodimers produced three-fold higher fluorescence intensity than either monomer alone.[39] The KSP*/QRH* heterodimer labeled with IRDye800CW is currently in a phase I clinical trial for the detection of neoplasia in the esophagus following topical application (Figure 3C).

One major limitation of intraoperative fluorescence imaging is the insensitive detection of buried cancer nodules, due to the limited penetration of light. This limitation can be overcome by developing dual-modal probes bearing both an NIR fluorescent dye and a radionuclide. While providing intraoperative real-time fluorescent images to visualize the tumor border in superficial tissues, the dual-modal probe can offer preoperative information on the location of tumor lesions by positron emission tomography (PET) or single photon emission computed tomography and intraoperative guidance of deep-laying nodules by gamma counting. Cirentuximab, a monoclonal antibody targeting carbonic anhydrase IX (CAIX), which is overexpressed in 95% of clear cell renal cell carcinoma, is labeled with IRDye800CW and 111In.[40] In the first-in-human study, intraoperative radiative guidance was shown to be essential for tumor localization in tissues covered by fat, since fat can significantly attenuate fluorescence signal. In addition, the dual-modal detection can help rule out false-positive lesions that are hyperfluorescent but not radioactive due to the autofluorescence of the cyst contents. With a similar strategy, a dual-modal probe based on the anti-CEA antibody labetuzumab was also developed for dual-modal imaging-guided surgery in colorectal cancer peritoneal carcinomatosis.[41] In addition to antibodies, peptides were also employed for this purpose. The bombesin (BBN) peptide, which targets the gastrin-releasing peptide receptor (GRPR), was labeled with 58Ga and IRDye800CW for PET/NIR fluorescence dual-modal imaging-guided surgery in glioblastoma (Figure 3D).[42]

OTL38 and ZW800

Although cyanine dyes containing a phenoxy ether-bridged rigid cyclohexyl moiety show increased fluorescence, one defect of such a structure is that the phenoxy ether bridge is prone to attack by an amine moieity, leading to unwanted byproducts and thereby low yields. Therefore, OTL38, an NIR fluorescent probe that binds FRs, was delicately designed to avoid this issue (Figure 3E). The glutamate moiety of folate was replaced with a tyrosinate moiety and then the phenoxy group reacts with the meso-chloride of S0456. This modification not only retained the targeting capability of folate and the brightness of phenoxy ether-bridged cyanine dyes, but also resulted in > 98% chemical yield and > 98% purity.[49] OTL38 has successfully completed phase 1 and phase 2 clinical trials for ovarian and lung cancers. In ovarian...
cancer patients, OTL38 at a dose of 0.0125 mg/kg enabled surgeons to identify malignant lesions with relatively high sensitivity (the mean of SBR was 4.4). Benefiting from the specific accumulation of OTL38 in tumor cells, surgeons can identify additional tumor tissues that cannot be observed by traditional detection. With its recent entry into phase 3 clinical trials, OTL38 is expected to be the first targeted fluorescent probe approved for fluorescence imaging-guided surgery in humans.

The chemical and geometric configurations play an important role in the nonspecific binding, uptake, and retention of NIR fluorophores. Choi et al. found that ZW800-1, a zwitterionic NIR fluorophore, is characterized by reduced nonspecific binding and rapid renal clearance (Figure 3F). Similar to IRDye800CW and OTL38, the core structure of ZW800-1 also includes a phenoxy ether-bridged rigid cyclohexyl moiety in the middle of the polymethylene chain. However, the net charge of ZW800-1 is zero and the geometrically balanced charges across the molecule shield the underlying hydrophobic heptamethine core. This unique structure endows ZW800-1 with fluorescent properties that are comparable to IRDye800CW, but reduced liver clearance and nonspecific binding. ZW800-1 also contains a carboxylic acid group for covalent conjugation to various targeting ligands. ZW800-1 provides a much improved SBR for targeted fluorescence imaging of tumors by conjugation with a cyclic RGD (cRGD) peptide. In a preclinical melanoma model, an SBR as high as 17.2 was achieved after intravenous injection of cRGD-ZW800-1 compared with SBRs of 5.1 for cRGD-IRDye800CW and 2.7 for cRGD-Cy5.5. cRGD-ZW800-1 is currently undergoing phase 2 clinical trial to investigate its feasibility of fluorescence-guided surgery in patients with oral cancer.

Other NIR fluorescent probes in the 800 nm window

Heat shock protein 90 (Hsp90) has been identified as a protein chaperone that maintains the proper folding of over 200 proteins. Despite the abundance and ubiquitous expression of Hsp90 in vivo, the level of extracellular Hsp90 (eHsp90) was found to be correlated with the malignancies of several tumor types, including breast cancer. Haystead et al. developed a series of fluorescent Hsp90 probes, consisting of a modified Hsp90 inhibitor, a PEG linker, and a hydrophilic fluorophore. The Hsp90-targeting ligand endows high binding affinity for Hsp90 and the hydrophilic nature of the fluorophore makes the probe cell-impermeable, resulting in exclusive binding to eHsp90. In addition, probe-bound eHsp90 can be actively internalized by tumor cells and the extent of internalization is related to tumor aggressiveness. Therefore, fluorescent Hsp90 probes can serve as targeted fluorescent probes to surgical navigation. HS-196, a fluorescent Hsp90 probe emitting in the 800 nm window (Figure 3G), is currently under clinical evaluation for the fluorescent detection of breast tumors.

BLZ-100 (Tumor Paint) is composed of the chlorotoxin (CTX) peptide covalently bound to a derivative of ICG (Figure 3H). The CTX peptide was originally discovered from scorpion venom and was found to specifically target several tumors through interaction with protein components in cholesterol-rich lipid rafts, including matrix metalloproteinase (MMP) 2. CTX was also shown to facilitate blood-brain-barrier penetration. BLZ-100 is currently undergoing a phase 3 clinical trial for intraoperative tumor detection in pediatric primary central nervous system tumors.

In most cases, the targeting ability results from the targeting ligand and not the fluorophore; however, Achilefu et al. discovered a novel dye-peptide conjugate that targets tumor cells through the synergistic effects of the NIR fluorophore cyrate and the peptide GRD. Since the RGD-containing peptide has been widely developed for 1 integrin-targeting, the GRD-containing peptide GRDSPK is presumably inactive. However, cyrate-GRDSPK conjugates unexpectedly accumulated in αvβ3 integrin-positive tumors, while neither cyrate-RGD nor the radiolabeled GRD peptide was retained in tumors. To further stabilize the peptide, an intramolecular disulfide bond with D-L cysteine was introduced, resulting in the targeted fluorescent probe LS301 (Figure 3I). A recent study found that LS301 selectively targets phosphorylated Annexin A2 (pANXA2), which is highly expressed in a range of solid tumors, including breast, pancreatic, and brain cancers. In addition, the free carboxylic acid group of cyrate can be utilized to conjugate with chemical drugs, such as doxorubicin, for drug-delivery applications.

Activatable fluorescent probes under clinical investigation

The fluorescent probes discussed above have a common problem in that they keep emitting fluorescence signals regardless of their interactions with the target tissues, leading to a considerable background signal interference. Compared with these “always-on” fluorescent probes, activatable fluorescent probes themselves are originally optically silent. They only emit significantly strong fluorescence in tumors taking advantage of the variations in tumors, including specific enzymes, pH, or redox potential.

AVB-620 was built on a pioneering work by Tsien et al. on activatable cell-penetrating peptide (CPP). Activatable CPP consists of a polycationic CPP and a polyamionic masking peptide, which are linked together through a protease substrate. The cellular uptake of activatable CPP is blocked by forming a hairpin structure through intramolecular electrostatic interactions. After cleavage of the linker by a protease, the CPP is released and the covalently attached payload are efficiently internalized by cells without requiring specific receptors. AVB-620 further optimized the sequence of an activatable CPP, and two fluorophores, Cy5 and Cy7, were conjugated to the cationic terminal and anionic terminal, respectively (Figure 4A). The unique hairpin structure allows for efficient fluorescence energy resonance transfer (FRET) between the two fluorophores. The peptide linker was designed to be cleaved by MMP2 and MMP9, which are highly expressed in human breast cancer cells. In addition, the αv-aminolyl-ω-methoxy PEG (mPEG, Mw~2000) moiety was conjugated to improve the water solubility. MMP-mediated hydrolysis disrupts FRET, and Cy5 is then internalized by cancer cells, leading to a large increase in the Cy5/Cy7 fluorescence ratio. The ratiometric readout is considerably less prone to optical artifacts, which are common in single intensity fluorescence imaging, including the distance from the tissues of interest to the camera and variations in the drug concentration. AVB-620 is currently the only clinical fluorescent probe utilizing ratiometric imaging. In the phase
FIGURE 4 Chemical structures of the activatable fluorescent probes. (A) AVB-620. (B) LUM015. (C) Representative tumor specimen from a patient after injection of LUM015. (D) Mechanism of fluorescence activation of GCP-001 and GCP-002. (E) Fluorescent images of lymph nodes from breast cancer patient: before (top), 5 min post (middle), and 15 min (bottom) post admonition of GCP-001. (F) The top image is the H&E staining of the same lymph node second from the left in (E). The bottom image is the magnified fluorescent image of the same lymph node. Reproduced with permission from: C, Ref. 60; E and F, Ref. 65
1 clinical trial, AVB-620 was demonstrated to be safe and allow for intraoperative detection of tumor-positive tissue. AVB-620 is currently undergoing a phase 2 trial to evaluate the accuracy of tumor detection in breast cancer surgery. Another protease-activated fluorescent probe in clinical trials is LUM015, which has a similar mechanism to that of AVB-620. LUM015 can be described as three major components linked to a GGRK peptide: a QSY21 quencher, a Cy5 fluorophore, and an mPEG moiety (Figure 4B). The GGRK peptide provides both cathepsin substrate specificity and proper spacing between the fluorophore and quencher. The QSY21 quencher is covalently attached to the N-terminus of the peptide, and the Cy5 fluorophore is attached to the ε-amino group of the lysine residue. In addition, mPEG moiety (Mw∼20,000) is conjugated to the C-terminus of the peptide via an aminoethoxyethoxyacetyl spacer, which separates the cleavage site from the mPEG chain to improve access to the peptide by cathepsins. The incorporation of the mPEG moiety significantly improves the solubility and pharmacokinetics of the probe. Intact LUM015 is optically inactive, but after cleaved by cathepsins, the quencher is released to generate fluorescent fragments. In a phase 1 study with 15 patients, LUM015 was well tolerated and could distinguish soft tissue sarcoma and breast cancer tissues from normal tissues in ex vivo surgery specimens. In another clinical trial, LUM015 was demonstrated to allow for real-time intraoperative identification of residual breast cancer with a mean SBR of 4.70 at a dose of 0.5 mg/kg.

Urano et al. developed several activatable fluorescent probes for topical applications based on hydroxymethyl rhodamine green (HMRG) (Figure 4C). One such probe is β-glutaryl HMRG (βGlu-HMRG, commercial name GCP-001). GCP-001 is completely quenched due to intramolecular spirocyclization but can be rapidly activated by one-step cleavage with β-glutamyltranspeptidase (GGT), which is overexpressed on the cellular surface of several tumors, including cervical and ovarian cancers. The hydrophilic GCP-001 probe cannot penetrate the cell membrane. However, once GGT is encountered on the surface of cancer cells, it is hydrolyzed to yield highly fluorescent HMRG, which is sufficiently hydrophobic for cell membrane penetration and accumulates mainly in lysosomes. The feasibility of GCP-001 for intraoperative fluorescence imaging was assessed with freshly excised human breast specimens. Topical spraying of GCP-001 allowed for the easy discrimination of breast tumors from normal tissues within only 5 min after application. Another important application of GCP-001 is rapid diagnosis of lymph node metastasis. In a study with 147 lymph nodes from 38 breast cancer patients, GCP-001 helps to visualize breast cancer cells in lymph nodes with high sensitivity (97%), specificity (79%), and negative predictive value (99%) (Figure 4E and F). Therefore, GCP-001 is currently undergoing a clinical trial in Japan to evaluate the efficacy for fluorescence detection of breast cancer lesions.

Esophageal cancer, in particular, esophageal squamous cell carcinoma (ESCC), is one of the most common causes of cancer-related death. However, GCP-001 is not suitable for ESCC detection. Therefore, based on the fact that various aminopeptidases are overexpressed in cancers, through screening, dipeptidylpeptidase IV (DPP-IV) was determined to be a suitable target for the detection of ESCC. By varying dipeptides conjugated to HMRG, EP-HMRG (commercial name GCP-002) was selected for the sensitive detection of ESCC. When topically sprayed onto fresh human ESCC specimens, tumors were visualized within 5 min and the sensitivity, accuracy, and specificity reached 96.9%, 90.5%, and 85.7%, respectively. Therefore, this DPP-IV-targeted activatable fluorescent probe might be a convenient tool for the accurate detection of esophageal cancer during surgical or endoscopic procedures.

Nanoscale fluorescent probes in clinical studies

In addition to the small-molecule fluorescent probes, another hotspot in this area is the development of nanoscale probes. Nanoscale probes are advantageous in several aspects. They can inherently target tumor tissues through the enhanced permeability and retention (EPR) effect, which is a common phenomenon in most solid tumors. The surface of the nanoparticles can also be modified with targeting ligands to achieve active targeting. In addition, therapeutic drugs or other imaging agents can be loaded into the fluorescent nanoprobes, thereby making theranostics or multimodal imaging possible. Most importantly, a number of fluorescent nanoprobes have significantly higher photostability and brightness than small-molecule probes. Currently, there are two nanoscale fluorescent probes undergoing clinical evaluation for fluorescence imaging-guided surgery. Particle size is a critical determinant of the pharmacokinetics of nanoprobes. A smaller size is an appealing feature for more uniform delivery of the probes in tumors. Nanoprobes with a size smaller than 10 nm exhibit an increased chance of rapid renal clearance and do not accumulate in the liver, which decreases potential toxicity and side effects. However, if the size is too small (smaller than 3 nm), an increasing amount of nonspecific diffuse might result in undesirable background fluorescence signals. Therefore, ultrasmall fluorescent core-shell silica nanoparticles (Cornell dots or C dots) were developed with a particle size of 6-7 nm (Figure 5A). These C dots were fabricated with a dye-encapsulated silica core coated with short PEG chains (Mw∼500) using the Stöber sol-gel process. The PEG coating creates a chemically neutral and bioinert surface, thereby reducing nonspecific cellular uptake and promoting renal clearance. Furthermore, iodine-124 (124I)-labeled cyclic RGDY (cRGDY) peptides were conjugated to the PEG chains, creating an integrin-targeting fluorescence/PET dual-modal probe (124I-cRGDY-PEG-C dot). Compared with free dyes in aqueous solution, the covalently encapsulated Cy5 dyes exhibit improved photostability and brightness (an over two-fold increase), due to an increased number of photons emitted after light excitation and a decreased number of photons lost to nonradiative process. In a first-in-human clinical trial, 124I-cRGDY-PEG-C dots were proven to be well-tolerated with renal clearance as shown by PET imaging. The uptake in normal organs indicated a favorable safety and biodistribution profile of the C dots. In addition, in preclinical melanoma models, C dots were found to accurately discriminate metastatic lymph nodes. C dots are currently undergoing clinical trials for the intraoperative mapping of metastatic lymph nodes via fluorescence imaging. C dots also serve as a versatile platform for various purposes,
such as molecular phenotyping and radiotherapy by varying the dyes, targeting ligands, and radionuclides.\cite{78}

Most solid tumors exhibit an acidic tumor microenvironment (pH = 6.5-6.9), which has motivated researchers to develop a general-purpose fluorescent probe that is responsive to a decreased pH for imaging a wide range of tumors.\cite{79} ONM-100 is one such nanoprobe with a transistor-like response to pH (transition pH = 6.9). It is a micellar probe consisting of ICG-derivative-labeled poly(ethylene glycol)-b-poly(ethylpropylaminoethyl methacrylate) copolymers (PEG-b-P(EPAX-r-ICG\textsubscript{y})).\cite{80} By adjusting the length of poly(ethylpropylaminoethyl methacrylate) (PEPA) and the number of conjugated ICG moieties, PEG-b-P(EPAX\textsubscript{100-r}-ICG\textsubscript{1}) was chosen as the optimal composition for a higher fluorescence activation ratio and a sharper pH transition. Above pH 6.9, hydrophobic micellization induces the formation of nanoscale micelles, and the fluorescence of ICG is quenched. Below pH 6.9, the micelle dissociates into unimers and...
fluorescence is recovered. Therefore, ONM-100 acts as a pH binary on/off switch to amplify the acid pH in tumor microenvironment, while suppressing signal in blood (pH 7.4) (Figure 5B). In preclinical models, ONM-100 was well-tolerated and in all groups, tumor tissues showed higher fluorescence than the surrounding tissues with a median SBR of 4.5, which confirmed the effectiveness and tolerance of ONM-100.\(^{[81]}\)

**Emerging NIR-II fluorescent probes in the preclinical stage**

Although fluorescence imaging in the NIR-I region is more favorable than that in the visible range due to reduced photon scattering and tissue autofluorescence, increasing researches have shown that the clarity, penetration depth, and SBR of fluorescence imaging can be further improved by extending the wavelength beyond 1000 nm. This can be explained by the minimal photon scattering and autofluorescence in the NIR-II region. Compared with NIR-I imaging, in vivo NIR-II fluorescence imaging has relatively less explored until significant technique advances in longer-wavelength indium–gallium–arsenide (InGaAs) cameras and biocompatible NIR-II emissive fluorophores were made in recent years.\(^{[82]}\) Cameras in most fluorescent imaging systems are based on silicon detectors, whose longest detectable wavelength is typically below 1000 nm. The commercial availability of InGaAs cameras with the ability to detect NIR-II light has boosted the rapid growth of NIR-II fluorescence imaging. In addition, several novel NIR-II emissive biocompatible fluorophores, such as carbon nanotubes, metal chalcogenide QDs, RE-doped nanoparticles, and D-A-D dyes, have been fabricated and explored for their biomedical applications in recent years. A recent first-in-human study of NIR-II imaging with ICG indicated a significantly higher SBR than that of NIR-I imaging (5.33 versus 1.45) for the intraoperative detection of primary and metastatic liver tumors.\(^{[83]}\) Therefore, although most fluorescent probes under clinical evaluation are intended for NIR-I imaging, we can envision a gradual transition to NIR II imaging in the near future.

**Carbon nanotubes for NIR-II imaging**

The initial reports of intravital fluorescence imaging in the NIR-II window were conducted with PEG-functionalized single-walled carbon nanotubes (SWCNTs) by Dai et al.\(^{[84]}\) SWCNTs are hydrophobic and exhibit NIR-II fluorescence due to van Hove transitions across bandgaps when dispersed in solution (Figure 6A). Surface functionalization with PEG-grafted lipids not only improves the fluorescence quantum yield of SWCNTs, but also renders SWCNTs biocompatible for in vivo applications. Since then, SWCNT-based probes have been demonstrated for dynamic contrast-enhanced NIR-II imaging through principal component analysis (PCA) (Figure 6B),\(^{[85]}\) tumor-specific imaging,\(^{[86]}\) and hemodynamic imaging of mouse peripheral vessels.\(^{[87]}\) By subdividing the NIR-II fluorescence of SWCNTs into the 1300–1400 nm window, through-skull fluorescence imaging of the mouse brain was achieved for the first time, with a depth of > 2 mm and sub-10 μm resolution.\(^{[88]}\) In addition, SWCNTs with large diameters synthesized by laser vaporization exhibited high fluorescence in the NIR-Iib (1500-1700 nm) window.\(^{[89]}\) With these imaging agents, NIR-Iib fluorescence imaging enabled intravital vascular imaging with a spatial resolution as low as 4 μm at a depth of 3 mm in mouse hindlimbs and brains with an intact skull and scalp. Furthermore, tumor-targeted NIR-II imaging can be achieved by modifying SWCNTs with tumor-targeting ligands. For example, SWCNTs were stabilized with the M13 virus, and the filamentous M13 virus (M13-SWCNTs) was genetically modified with a targeting peptide.\(^{[90]}\) Targeted ovarian cancer imaging with this nanoprobe exhibited a higher SBR and detected more tumor nodules compared with visible and NIR-I dyes.

**NIR-II quantum dots**

Despite the initial success, the polydispersed size distribution and low quantum yield of SWCNTs have hampered their further biomedical applications. Therefore, vast efforts have been devoted to the development of novel NIR-II emissive probes. Among the probes reported thus far, NIR-II QDs, such as PbS, Ag\(_2\)S, Ag\(_2\)Se, and InAs, exhibit the highest fluorescence quantum yields (up to 30%).\(^{[90]}\) The Ag\(_2\)S QD, which does not contain toxic heavy metal ions, was the first NIR-II emissive QD and is perhaps the most promising for clinical translation.\(^{[91]}\) After modification with branched 6-arm PEG, Ag\(_2\)S QDs accumulate significantly in tumors via the EPR effect (Figure 6C).\(^{[92]}\) Ag\(_2\)S QDs significantly outperform fluorophores in the visible and NIR-I windows for visualizing lymph nodes, tissue blood flow, and angiogenesis (Figure 6D).\(^{[93]}\) In addition, the Ag\(_2\)S QDs can also serve as a multifunctional platform for fabricating activatable NIR-II fluorescent probes.\(^{[94]}\) Due to the potential of Ag\(_2\)S QDs for clinical applications, Ag\(_2\)S QDs have been extensively investigated to further improve their optical properties.\(^{[95]}\) For example, a recent work by Santos et al. presented a novel methodology that increases the quantum yield of Ag\(_2\)S QDs by 80-fold.\(^{[96]}\) This process is conducted by irradiating chloroform-dispersed Ag\(_2\)S QDs with a femtosecond laser, which forms a protective AgCl shell. This shell reduces structural defects and thereby increases the fluorescence quantum yield.

Another promising NIR-II QDs reported is the gold nanoclusters. Liu et al. presented an atomic-precision gold nanocluster with 25 gold atoms and 18 peptide ligands, showing emission at 1100-1350 nm and increased fluorescence quantum yield by metal-atom doping.\(^{[97]}\) One of the most attractive characteristics is the ultrasmall size, allowing rapid renal clearance. The glutathione-capped gold nano-clusters were also found to accumulate in bone tissues, rendering their great potential for fluorescence-guided surgery like spinal pedicle screw implantation.\(^{[98]}\) Song et al. also reported an NIR-II gold nanocluster protected by cyclodextrin (CD).\(^{[99]}\) The macrocyclic CD on the surface of gold nanoclusters not only renders good stability and efficient renal clearance, but also allows facile protein labeling via the host-guest chemistry.
By labeling with gold nanoclusters with anti-CD326 antibody, the accumulation of gold nanoclusters in tumors was enhanced by ~three-fold at 24 h post injection the subcutaneous breast cancer model. Moreover, the protein-labeled gold nanoclusters can also undergo efficient renal clearance.

**RE-doped NIR-II nanoparticles**

Another class of NIR-II fluorophores are RE-doped nanoparticles, which are composed of lanthanide ions embedded in an inorganic crystalline host matrix (e.g., NaGdF₄, NaYbF₄, and NaYF₄). The NIR-II fluorescence of RE-doped nanoparticles is a result of resonant energy transfer from a sensitizer to an RE activator dopant (e.g., Er, Tm, Ho, Nd, and Pr), whose relaxation produces a unique activator-dependent downconversion (DC) emission in addition to the common upconversion (UC) emission. A typical NIR-II emissive RE-doped nanoparticle is composed of an Er/Yb co-doped crystalline core surrounded by an inert shell.[100] The Yb³⁺ ions absorb 980 nm light and then transfer this energy to excite Er³⁺, which can relax to produce NIR-II DC emission beyond 1500 nm. The NIR-II DC emission may be compromised by the UC emission and quenching of excited Er³⁺ in aqueous solution. To address this issue, Ce doping in the core was found to inhibit the UC pathway to boost DC emission by nine-fold, while aqueous quenching can be alleviated by optimizing the thickness of the inert shell.[101] Most of the reported Er-based nanoparticles are in β-phase (hexagonal phase).[102] A recent study described α-phase (cubic phase) Er-based nanoparticles with an 11-fold enhancement in DC emission over the previously found...
brightest β-phase Er-based nanoparticles (Figure 6E).\textsuperscript{103} This ultrabright nanoparticle was coated with crosslinked hydrophilic polymers and then functionalized with an anti-programmed cell death 1 ligand 1 (anti-PD-L1) antibody for in vivo molecular imaging. In a mouse colon cancer model, an SBR as high as ~40 was achieved. Another important characteristic of RE-doped nanoparticles is that their luminescence lifetime can be delicately manipulated from the microsecond to millisecond range by tuning the nanoparticle atomic makeup. Luminescence lifetime imaging allows for the simultaneous imaging of multiple targets in vivo independent of tissue penetration depth, which is unachievable with conventional multicolor imaging.\textsuperscript{103,104} Despite the advantages of RE-doped nanoparticles with respect to their excellent optical properties, caution in the choice of stable surface coatings needs to be taken to render them biocompatible before they can be used in humans.

**NIR-II imaging utilizing off-peak emission of NIR-I cyanine dyes**

Recent spectral investigations with InGaAs detectors of NIR-I cyanine dyes, including ICG and IRDye800CW, revealed long emission tails that extend into the NIR-II region (even beyond 1500 nm), opening an entirely new route for clinical NIR-II fluorescence imaging (Figure 7A and B).\textsuperscript{105,106} The underlying mechanism of NIR-II emission was identified as the twisted intramolecular charge transfer (TICT) process from the asymmetry of the π domain in the S\textsubscript{1} state of NIR-I cyanine dyes.\textsuperscript{107} Even though their emission spectra peaks are in the NIR-I region, these NIR-I cyanine dyes outperform early-generation NIR-II dyes (eg, IR-E1050). NIR-II fluorescence imaging with ICG at ~1300 nm increased brain vasculature contrast by nearly 50% and decreased the apparent vessel width from 430 to 210 μm compared with traditional NIR-I fluorescence imaging (Figure 7C and D).\textsuperscript{105} The TICT S\textsubscript{1} state is very sensitive to the surrounding environment and NIR-II emission increases significantly when bound to serum albumin.\textsuperscript{108} Tian et al. engineered a supramolecular assembly of IR-783 and bovine serum albumin (BSA).\textsuperscript{109} The complexation of BSA with IR-783 can hold the twisted conformation and enhance the TICT process, providing an NIR-II quantum yield of 21.2% with prolonged circulation time. Due to the well-established synthetic routes and safety profiles of NIR-I cyanine dyes that have been demonstrated in several clinical studies, further exploration of off-peak NIR-II emission and the pharmaceutical development of bright NIR-II fluorophores based on NIR-I cyanine dyes may accelerate the clinical translation of NIR-II fluorescence imaging.\textsuperscript{110}

**NIR-II emissive cyanine dyes**

In addition to utilizing the off-peak tail emission of NIR-I cyanine dyes, redesigning the traditional cyanine dyes to red-shift their peak emission into the NIR-II region is another research hotspot. Increasing the length of the polymethine chain, a classical method to redshift cyanine dyes as mentioned above, can compromise the fluorescence quantum yield and stability.\textsuperscript{111} Therefore, modifying the terminal heterocyclic groups has emerged as an alternative strategy, which has been utilized to fabricate a few commercially available polymethine dyes, such as IR-26 (Figure 7E), IR-1061, and IR-1048. These initial successes inspired researchers to fabricate novel NIR-II cyanine dyes with improved fluorescence quantum yield and biocompatibility. In an attempt by Cosco et al., the sulfur atom of IR-26 was replaced with oxygen to increase the fluorescence quantum yield, and electron-donating dimethylamino groups were added to compensate for the blueshifted absorbance caused by the oxygen (Figure 7F).\textsuperscript{112} The resulting flavylum polymethine fluorophore Flav7, with a peak absorbance and emission over 1000 nm, shows a quantum yield of 0.53 (IR-26 = 0.05%). In another attempt by Wang et al., the heptamethine chain of IR-26 was changed to the shorter pentamethine chain, and electron-donating diethylamino moieties were also adopted for bathochromic shifts (Figure 7G).\textsuperscript{113} Thanks to the shorter polymethine, the resulting BCT1070 dye exhibits significantly reduced solvatochromism-caused quenching in polar solvents, thus allowing peak absorption/emission beyond 1000 nm with ~seven-fold enhanced brightness relative to IR-26 and superior photostability in aqueous solution. Another interesting study by Li et al. reported a novel NIR-II emissive cyanine dye, FD-1080, with a similar structure to that of ICG (Figure 7H). The quantum yield of FD-1080 is 0.31% (IR-26 = 0.05%) and can be increased to 5.94% in serum. FD-1080 was found to form J-aggregates by self-assembly with 1,2-dimyrystoyl-sn-glycerol-3-phosphocholine (DMPC).\textsuperscript{114} Compared with the FD-1080 monomer, the absorption/emission of FD-1080 J-aggregates was bathochromically shifted by approximately 300 nm, thus affording superior spatial resolution and a high SBR for NIR-II vascular imaging beyond 1500 nm.

**Donor-acceptor-donor dyes for NIR-II imaging**

Another promising NIR-II emissive fluorophore is the D-A-D dye with highly tunable emission in the NIR-II region. A typical D-A-D dye is CH1055 developed by Antaris et al. (Figure 7I).\textsuperscript{115} This CH1055 dye is composed of a central benzo[1,2-c:4,5-c’]bis[1,2,5]thiadiazole (BBTD) moiety, a strong electron withdrawing group, flanked by two triphenyl amino groups as electron donors. In addition, four carboxylic acid groups were introduced into the CH1055 architecture to impart aqueous solubility and allow for the facile conjugation to various functional moieties. By conjugating PEG moieties with CH1055, the resulting CH1055-PEG exhibited rapid renal clearance, high passive tumor uptake, and excellent performance in vascular imaging and SLN mapping. The quantum yield of CH1055-PEG is 0.03% (IR-26 = 0.05%). In an attempt to further enhance the fluorescence quantum yield, CH1055 was sulfonated utilizing the four carboxylic acid groups (CH-4T).\textsuperscript{116} The quantum yield of CH-4T in fetal bovine serum (FBS) increased to 0.48%, and further heating to 70°C for 10 min increased the fluorescence quantum yield to 1.08%.

Due to the large conjugated backbone, NIR-II organic fluorophores tend to have strong intermolecular interactions, and the excited states of the molecules are prone to attack and quenching. Therefore, the low quantum yield of NIR-II organic fluorophores, such as CH1055, was attributed to interactions with water.\textsuperscript{117} To address this issue, a common
FIGURE 7  (A) The fluorescence spectrum of ICG measured with InGaAs detector and the absorption spectra. (B) The magnified emission spectra of ICG in the NIR-II region. (C) NIR-I fluorescence imaging of brain vasculature of a mice with ICG using the 850-nm long-pass (LP) filter. (D) NIR-II fluorescence imaging with 1300-nm LP filter. Chemical structure of (E) IR-26, (F) Flav7, (G) BTC1070, (H) FD-1080, and (I) CH1050. (J) Schematic illustration of the p-FE fabrication. (K) Two-color NIR-II fluorescence imaging of 4T1 tumor. SWCNTs were first injected and then accumulated in the tumor via EPR effect. The p-FE was then injected to highlight vascular structure. Under the excitation of 808 nm laser, the p-FE signal was collected with 1200-1300 nm filter, while the SWCNT signal was obtained with 1500-1700 nm filter. Figures reproduced with permission from: A, B, C, D, Ref. 105; J, K, Ref. 120
strategy is to introduce shielding units (Ss) on both sides of D-A-D dyes, forming S-D-A-S variants.\textsuperscript{118,119} Wan \textit{et al.} constructed the bright nanoprobe p-FE based on an S-D-A-S dye (FE) (Figure 7J).\textsuperscript{120} This FE is constructed with BBTD as the acceptor, 3,4-ethyleneoxyxiphophene (EDOT) as the donor, and dialkyl fluorene as the shielding unit. EDOT can distort the backbone and afford a less delocalized lowest unoccupied molecular orbital. In addition, the alkyl chains stretching out of the plane of the conjugated backbone can prevent the backbone from aggregating from the FE. The quantum yield of the resulting p-FE was estimated to be up to 1.65\% (IR = 0.05\%). In vivo, two-color fluorescence imaging was successfully performed utilizing p-FE and SWCNTs to label the vasculature and tumor separately (Figure 7K).

KEY CONSIDERATIONS IN THE DESIGN OF FLUORESCENT PROBES FOR CLINICAL TRANSLATION

Optical properties of fluorophores

As shown in the previous section, the performance of intravital fluorescence imaging can be enhanced with increasing excitation/emission wavelength. Most current clinical studies are focused on NIR-I imaging. The most prevalent NIR-I fluorophores in clinical trials use heptamethine cyanine dyes containing a phenoxy ether-bridged rigid cyclohexyl moiety, such as IRDye800CW, OTL38, and ZW800. Although the central ether bridge is labile during amide-coupling chemistry and circulation in the bloodstream, fluorophores with such core structures are advantageous for producing higher fluorescence quantum yield and brightness (brightness = extinction coefficient × fluorescence quantum yield).\textsuperscript{121} Brighter fluorescent probes enable many exciting possibilities for medical imaging in terms of exposure time, penetration depth, and imaging speed.\textsuperscript{116,120} In addition, as toxicity is inevitable for any injected fluorescent probe, fluorescence imaging with brighter fluorescent probes can be conducted at a lower dose. Therefore, improving the brightness of the fluorophores is key in designing novel fluorescent probes for clinical translation.

One of the most common strategies to improve the fluorescence quantum yield is to form a dye-protein complex utilizing the binding affinity of specific fluorophores with serum proteins. One typical example is ICG. When bound to serum proteins, the NIR-I fluorescence quantum yield of ICG can be increased by nearly 3.5-fold.\textsuperscript{122} This phenomenon is particularly evident for fluorophores emitting in the NIR-II region, such as the 110-fold fluorescence increase in CH-4T and ~19-fold fluorescence increase in FD-1080 when exposed to FBS and the 21-fold increase in NIR-II fluorescence of IR820 when complexed with human serum albumin.\textsuperscript{108,116,125} The underlying mechanism should be the reduced aggregation by protein binding and minimized nonradiative decay by the rigid conformation of the dye. Although fluorescence brightness can be improved by protein binding, such enhancement might be at the expense of a hampered excretion profile. For example, while PEG-CH1055 is rapidly cleared renally, CH-4T can be retained in the liver for over 1 month.\textsuperscript{116} In addition, fluorophores with high protein binding affinity may also result in a high background signal. Fluorescent probes with ultralow protein binding can account for a highly enhanced SBR (eg, ZW800-1).\textsuperscript{46} Therefore, caution needs to be taken with respect to the pharmacokinetics and nonspecific binding when considering the clinical translation of dye-protein complexes.

Another strategy to improve fluorescent quantum yield emerges in recent years is to utilize the aggregation-induced emission (AIE), inspired by the pioneering work by Tang.\textsuperscript{124} AIE mechanism is attracting increasing attention, mainly owing to its unique characteristic of suppressing the unwanted aggregation-caused quenching effect, which exists in many dyes, including cyanine dyes. The AIE dyes are generally fabricated with an aromatic rotors or twisted conformation. The AIE mechanism was considered to be the suppression of intramolecular rotations or vibration.

Increasing research has shown that nanosized fluorophores are more advantageous in terms of brightness, photostability, and availability for multiple functions. By immobilizing organic dyes in the nanocarrier, the fluorescence quantum yield can be enhanced by virtue of restricting intramolecular motion, which has been utilized for the development of the first translatable fluorescent nanoprobe, the C dot.\textsuperscript{73} This is also particularly useful for NIR-II emissive fluorophores, which emit fluorescence that relies on the distorted conformation.\textsuperscript{119,125} Moreover, the large hydrophobic π-conjugated systems cause the fluorophores to be prone to aggregation in aqueous solution, and the stability of such fluorophores can be enhanced by forming nanoparticles.\textsuperscript{120} In addition to organic fluorophores, inorganic fluorophores, such as semiconducting QDs and RE-doped nanoparticles, exhibit several unique characteristics, including increased brightness, low photobleaching, and a tunable luminescence lifetime. However, there are still many issues to be addressed for the clinical translation of newly developed nanoparticles, such as biosafety and large-scale manufacturing, which will be discussed in the following sections.

Recognition motif for tumor targeting

Targeted fluorescent probes can target tumors via two primary strategies: (1) binding to specific molecular targets that are highly expressed in tumor cells or cancer-associated cells (ligand-targeted probes) and (2) fluorescence activation in response to variations in specific enzymes, pH, or redox potential of the tumor microenvironment (activatable probes).

Ligand-based fluorescent probes can be fabricated with antibodies, peptides, or small molecules as the tumor-targeting moiety. Thanks to their long half-life in vivo, antibodies labeled with fluorescent dyes allow intraoperative fluorescence imaging to be performed over a longer period of time postinjection.\textsuperscript{123} However, their relatively high cost of production and potential immunogenicity limit the applications of fluorescently labeled antibodies. In contrast, peptides and small molecules are advantageous with lower immunogenicity and easier manufacturing as a single compound with a definite structure.\textsuperscript{126} In addition, the relatively smaller size allows for deeper tissue penetration and rapid renal clearance. However, due to rapid clearance in the body, the
optimal time window for fluorescence imaging with peptide-and small molecule-based fluorescent probes is usually very short (a few hours).\textsuperscript{127} It is hard to conclude which type of ligand-based fluorescent probe is better, and their pros and cons should be weighed during their design depending on the particular needs.

Activatable generation of fluorescent signal contrast takes the advantage of specific endogenous stimuli in tumor microenvironment, such as reduced interstitial pH, changes in redox potential (mainly due to the higher glutathione concentration), or increased level of certain enzymes (such as MMPs and cathepsins). The activatable fluorescent probes are designed by converting the endogenous stimuli into a sharp change in fluorescent signal. The signal-conversion mechanism involved in the design of activatable fluorescent probes includes an FRET mechanism where the fluorescence ratio changes upon processing (eg, AVB-620) or a quenching mechanism where only the product is fluorescent (eg, LUM015 and GCP-001). Since the fluorescent signal is activated by internal stimuli in tumor, there is no need to remove the unreacted agents, while for ligand-based probes, the unbound probes need to be removed. One of the major issues of activatable fluorescent probes is that the small-molecule product of the enzymatic reaction can diffuse out of the site of interest. This obstacle can be overcome by charge-reversing (eg, AVB-620), attachment of large-molecular-weight PEG (eg, LUM015), or the reversing of hydrophobicity (eg, GCP-001).

**Improving the SBR**

The SBR is determined by the ratio of fluorescence signals in tumors versus nontumor tissues, which is a key for intraoperative tumor imaging. The SBR can be improved by optimizing the binding affinity of the tumor-targeting ligand or the enzymatic response of the cleavage substrate. The differences in the expression levels of the selected targets between tumor and nontumor tissues significantly affect the SBR of the resulting targeted fluorescent probe. Therefore, identifying a molecular target with high selectivity is a prerequisite for fabricating an effective targeted fluorescent probe. Recognition elements of targeted fluorescence probes are usually based on the endogenous ligand or substrate of the specific receptor or enzyme. In addition, several screening approaches (eg, phage display) and direct optimization of the targeting ligands or enzymatic substrate can be used to improve the selectivity for cancer-specific targets.

In addition, an increasing number of preclinical and clinical studies have shown that fluorescence imaging in the NIR region has a higher SBR than that in the visible range, and the SBR increases with wavelength due to reduced light scattering and tissue autofluorescence.\textsuperscript{10,83,86} Due to the inhomogeneity of the refractive indices of different components of animal tissues, excitation photons are reflected during tissue penetration. Although the exponent for wavelength dependence differs, the declining trends for all the scattering curves in different tissues indicate reduced scattering at longer wavelengths. In addition, there are many endogenous fluorophores that emit in the visible range that accounts for the high background autofluorescence. The autofluorescence spectra of animal tissues exhibit a slowly decaying curve that ultimately declines into a virtually zero tissue autofluorescence region beyond 1500 nm.\textsuperscript{128} The reduced light scattering and tissue autofluorescence at longer wavelengths directly contribute to the higher SBR. In research on targeted ovarian cancer imaging with M13 viruses, the M13 viruses were either complexed with SWCNTs or labeled with AF750 dyes or FITC, and the probes were combined for injection into tumor-bearing mice.\textsuperscript{186} The tumor-to-muscle ratio of NIR-II imaging with SWCNTs was 5.5±1.2, which was significantly higher than that of NIR-I imaging with AF750 (3.1±0.42) and visible imaging with FITC (0.96±0.10).

Another aspect for improving the SBR is to reduce the nonspecific binding of the targeted fluorescent probes. Most NIR emissive dyes contain a large hydrophobic conjugated backbone, and hydrophobicity can lead to serious nonspecific protein binding. Improving the overall hydrophilicity through sulfonation of the dyes can reduce the protein binding to some degree (eg, IRDye800CW and OTL38). However, the negative charges of the sulfonic acid groups can also induce protein binding. Dyes with a well-balanced charge and a net surface charge of 0 (eg, ZW800-1) were found to allow low nonspecific binding and thereby increase the SBR when used for tumor-targeted molecular imaging. Surface grafting of PEG moieties is another common strategy to reduce nonspecific binding, especially for nanoparticles. The level of protein binding is related to the length, density, and configuration of the PEG moiety.\textsuperscript{129} In general, a dense coating of high-molecular-weight PEG in brush mode is preferred for low protein binding. In addition, alternatives for stealth coatings, including surface modification with zwitterionic polymers or peptides, have been demonstrated to have a better capability to reduce nonspecific binding.\textsuperscript{130}

**Route of administration**

There are three main routes of administration for fluorescent probes: intravenous injection, subcutaneous injection, and topical administration. The most common route of administration of fluorescent probes is intravenous injection. Following intravenous injection, fluorescent probes can unbi- edly interact with almost all tissues and highlight deep-laying structures, thus directing surgeons to lesions that cannot be seen by the naked eye.\textsuperscript{10} To achieve enough exposure to the injected probes in the targeted tissues of interest, the dose for intravenous injection is usually the highest among all routes of administration. In addition, since the whole body is exposed to the injected probe, the long-term retention and safety in major organs need to be well studied before considering clinical translation, which will be discussed in the next section.

Subcutaneous injection of fluorescent probes near the primary tumor is usually used for SLN mapping. The SLN is the first lymph node that drains cancer cells. If the SLN is normal, the echelon lymph nodes are unlikely to be affected. Therefore, the status of SLN is an important prognostic implication in determining surgical management and the need for adjuvant chemotherapy after surgery. The basis of SLN localization is that probes subcutaneously injected close to the primary tumors can be drained into the SLN. Nontargeted fluorescent dyes, such as ICG and MB, have been used in the clinic for SLN mapping. In addition, targeted
fluorescent probes have been found to accurately discriminate metastatic lymph nodes (eg, C dots). The size of the fluorescent probes significantly affects their retention in lymph nodes. Nanopores with a size smaller than 15 nm can be cleared within 48 h, while larger nanopores (> 50 nm) can be retained in the lymph nodes for several weeks.

Another route of administration is topical administration, which has been proven to be feasible for the fluorescent detection of superficial lesions, such as oral cancer, esophageal cancer, and colorectal cancer. Compared with intravenous injection, topical administration allows for rapid identification of neoplastic lesions at a very low dose, reducing systemic exposure and potential toxicity. However, due to the limited penetration depth, topically administered probes can only highlight the structures at the surface where they are applied, and tumor detection might be detrimentally affected by mucus consistency and bowel cleanliness.

Since activatable fluorescent probes are nonfluorescent without enzymatic cleavage, there is no need to wash out the unreacted agents. However, for ligand-based probes, the unbound probes need to be removed by washing with aqueous solution after topical administration.

Safety concerns

In addition to ensuring the efficacy of targeted fluorescence imaging, product safety needs to be well assessed in terms of biodistribution, clearance, metabolism, and toxicology. Since most fluorophores are not biodegradable, designing fluorescent probes with optimal clearance properties will minimize toxicity by reducing the duration of exposure. Renal clearance is a desirable pathway for rapidly removing injected agents and minimizing side effects resulting from potential catabolism or breakdown in the human body. The process of renal clearance involves glomerular filtration, tubular secretion, and finally elimination via urinary excretion. Glomerular filtration is highly size-dependent, and a hydrodynamic diameter (HD) less than 6 nm is preferred for filtration, while particles with an HD > 8 nm typically cannot undergo glomerular filtration. In addition to size, nonspecific protein binding is another factor that affects renal clearance, as protein adsorption may increase the HD. As discussed above, improving the overall hydrophilicity and charge balance of the dyes can reduce protein binding and thereby improve the efficiency of renal clearance (eg, IRDye800CW, OTL38, and ZW800-1). Surface grafting of PEG moieties on ultrasmall nanopores is feasible to allow the nanopores to be cleared renally (eg, C dots). However, PEG modification can also dramatically increase the HD of the nanopores, thereby reducing renal filtration. An alternative strategy is to coat the nanopores with zwitterionic small molecules, which was shown to be an effective way to fabricate renal-clearable QDs.

Hepatic clearance is the primary pathway of excretion for fluorescent probes that are unable to be removed in the kidney. Hepatic clearance is a much more complex process than renal clearance. The retention time of fluorescent probes in the liver varies from a few hours to several months, depending on the size, charge, composition, and surface modifications. A short retention time in the liver is desired for translatable fluorescent probes. All foreign materials excreted via the liver are catabolized first through hepatocytes. Therefore, a stable surface coating is needed to prevent the generation of toxic catabolites or heavy metal ions, especially for inorganic fluorescent probes, such as RE-doped nanoparticles and semiconducting QDs.

Manufacturing process

To translate the targeted fluorescent probes, it is necessary to produce enough probes to support clinical applications in hundreds to thousands of patients with high yield and purity. The manufacturing process should be performed following cGMP principles. Class 1 solvents need to be carefully avoided, and any impurities need to be decreased to an acceptable level. In addition, batch-to-batch differences in large-scale manufacturing need to be well controlled. This is a hard task. Therefore, although a number of targeted fluorescent probes have exhibited promising results in preclinical studies, their usually sophisticated designs may complicate their potential pharmaceutical development in terms of the manufacturing process, quality control, and reproducibility.

CONCLUSION

As we have shown in this review, the last decade has seen the rapid development of intraoperative fluorescence imaging, and the potential benefits of targeted fluorescent probes have become clear. With the development of novel fluorescent probes with improved physicochemical properties and tumor-targeting capabilities, surgeons can intraoperatively delineate tumor margins and detect residual tumors more accurately. These advances will revolutionize tumor surgery by maximizing resection efficiency and improving survival outcomes. Initial success has been reached with the clinical translations of EC-17, IRDye800CW, and OTL38. Additional fluorescent probes, including activatable probes and nanoscale probes, have also entered the phase of clinical evaluation. In addition, increasing research has recently demonstrated significant advantages of novel fluorophores that emit in the NIR-II region (900-1700 nm), such as SWCNTs, metal chalcogenide QDs, RE-doped nanoparticles, and D-A-D dyes. Owing to further reduced photon scattering and tissue autofluorescence, fluorescence imaging in the NIR-II region has shown a dramatic increase in clarity, penetration depth, and the SBR compared with NIR-I imaging. Therefore, although current clinical studies are focused on fluorescence imaging in the NIR-I region, we can envision a gradual transition to NIR-II imaging. Since the clinical translation of a new fluorescent probe is not easy, it is of great importance to learn from existing probes.

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