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Target-Cancer-Cell-Specific Activatable Fluorescence Imaging Probes: Rational Design and in Vivo Applications

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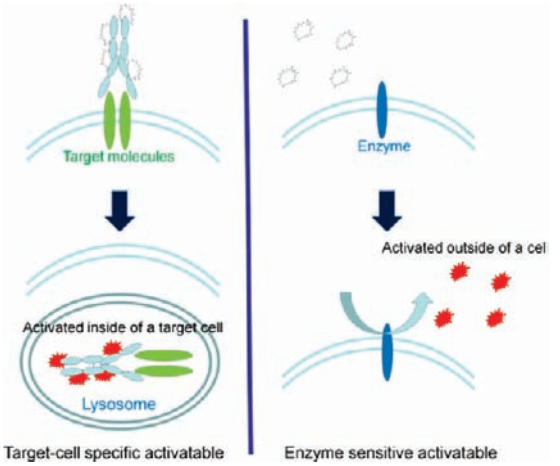
CONSPECTUS

Conventional imaging methods, such as angiography, computed tomography (CT), magnetic resonance imaging (MRI), and radionuclide imaging, rely on contrast agents (iodine, gadolinium, and radioisotopes, for example) that are "always on." Although these indicators have proven clinically useful, their sensitivity is lacking because of inadequate target-to-background signal ratio. A unique aspect of optical imaging is that fluorescence probes can be designed to be activatable, that is, only "turned on" under certain conditions. These probes are engineered to emit signal only after binding a target tissue; this design greatly increases sensitivity and specificity in the detection of disease.



Current research focuses on two basic types of activatable fluorescence probes. The first developed were conventional enzymatically activatable probes. These fluorescent molecules exist in the quenched state until activated by enzymatic cleavage, which occurs mostly outside of the cells. However, more recently, researchers have begun designing target-cell-specific activatable probes. These fluorophores exist in the quenched state until activated within targeted cells by endolysosomal processing, which results when the probe binds specific receptors on the cell surface and is subsequently internalized. In this Account, we present a review of the rational design and in vivo applications of target-cell-specific activatable probes.

In engineering these probes, researchers have asserted control over a variety of factors, including photochemistry, pharmacological profile, and biological properties. Their progress has recently allowed the rational design and synthesis of target-cell-specific activatable fluorescence imaging probes, which can be conjugated to a wide variety of targeting molecules. Several different photochemical mechanisms have been utilized, each of which offers a unique capability for probe design. These include self-quenching, homo- and hetero-fluorescence resonance energy transfer (FRET), H-dimer formation, and photon-induced electron transfer (PeT). In addition, the repertoire is further expanded by the option for reversibility or irreversibility of the signal emitted through these mechanisms.

Given the wide range of photochemical mechanisms and properties, target-cell-specific activatable probes have considerable flexibility and can be adapted to specific diagnostic needs. A multitude of cell surface molecules, such as overexpressed growth factor receptors, are directly related to carcinogenesis and thus provide numerous targets highly specific for cancer. This discussion of the chemical, pharmacological, and biological basis of target-cell-specific activatable imaging probes, and methods for successfully designing them, underscores the systematic, rational basis for further developing in vivo cancer imaging.

1. Introduction

The contrast agents used in conventional imaging, such as computed tomography (CT), magnetic resonance imaging (MRI), and angiography, continuously emit signals and hence are "always on".[1] Even in the case of radionuclide imaging which can be specific for particular pathologic conditions, probes consist of a targeting moiety conjugated to an "always on" signaling payload.[2,3] The fundamental

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disadvantage of "always on" probes is that they emit signals regardless of their proximity or interaction with target tissues or cells, and as a result there is considerable background signal to contend with. In order to design superior molecular imaging probes, one seeks to either (1) maximize signal from the target, (2) minimize signal from the background, or (3) do both. Doing any of these leads to improved target-to-background ratio (TBR), which, in turn, improves sensitivity and specificity for detecting diseases with imaging. A long-term approach to maximizing signal from the target is based on altering biodistribution and pharmacokinetics of the imaging probe.[3] However, recent advances in activatable agents that emit signal after enzymatic digestion,[4,5] or acidification,[6,7] represent an exciting strategy for improving target to background ratios.

Optical imaging with fluorescent probes is a relatively new medical imaging technique. It has advantages of lower cost, portabililty, and real time capabilities. Of course, the limited depth of light penetration in tissue necessitates that fluorescence optical imaging be reserved for surfaces such as those encountered during a multitude of endoscopic or surgical procedures. A unique feature of fluorescence is that optical probes can be designed so that they do not emit light, if they are unbound, while they only generate light upon internalization. This attribute of "turning on" only at the target has been termed activatable or "smart". Therefore, activatable agents have low to no background signal and generate signal only after being bound to the specific molecular target.[8,9] These activatable fluorescence probes maximize the target signal while minimizing the background signal, thereby resulting in higher target-to-background ratios than conventional "always on" imaging agents (Figure 1).

There are several ways to design activatable fluorescence probes. The typical method involves enzymatic activation of the probe by secreted extracellular or cell surface enzymes. In this strategy, the probe is silent until it is activated by specific microenvironments. Enzymatic cleavage then leads to generation of fluorescence signal mostly in the extracellular space.[9,10] The second strategy involves target-cell-specific activatable probes, which are quenched until activated within targeted cells by lysosomal processing of the probe. This occurs when the probe binds to the target cell surface molecule, which leads to subsequent internalization of the probe and fluorescence activation through chemical processes of the fluorescent probe within the endolysosome. This strategy results in intracellular signal activation-only in the target cells (Figure 2).[11,12]

There are advantages and disadvantages to both methods. In enzymatic activation, a single target enzyme can activate many fluorescence molecules, thus amplifying the signal from the target tissue. However, a disadvantage of enzymatic activation is that the activation occurs in the extracellular space. Therefore, after activation, the probe can diffuse away from the target and contribute to background signal. A few of the probes can be reacted with enzymes in the lysosome; however, the reaction mostly happens in the macrophage because of no cell selectivity

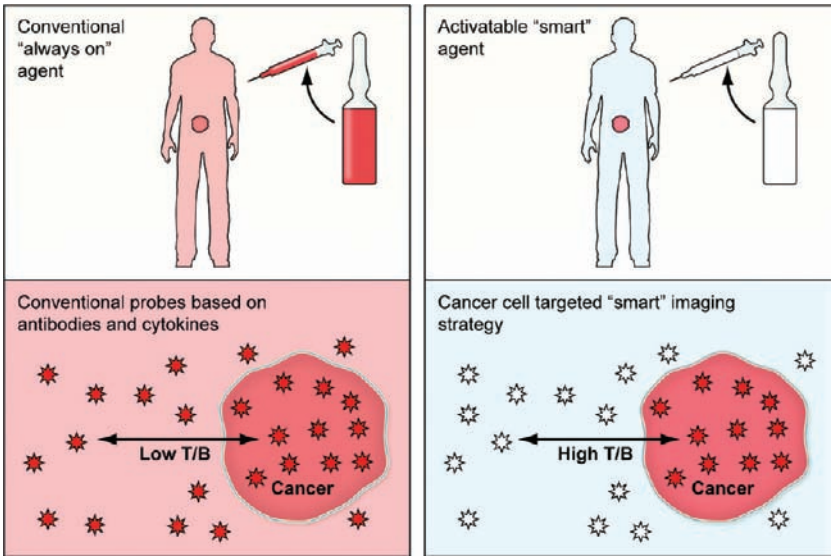

FIGURE 1. (Upper panel) Schema demonstrating targeted cancer imaging with a conventional "always on" probe (left) compared to a target-specific activatable probe (right). (Bottom panel) Schema demonstrating target signal-to-background ratio of a conventional "always on" probe (left) compared with a target-cell specific activatable probe (right).

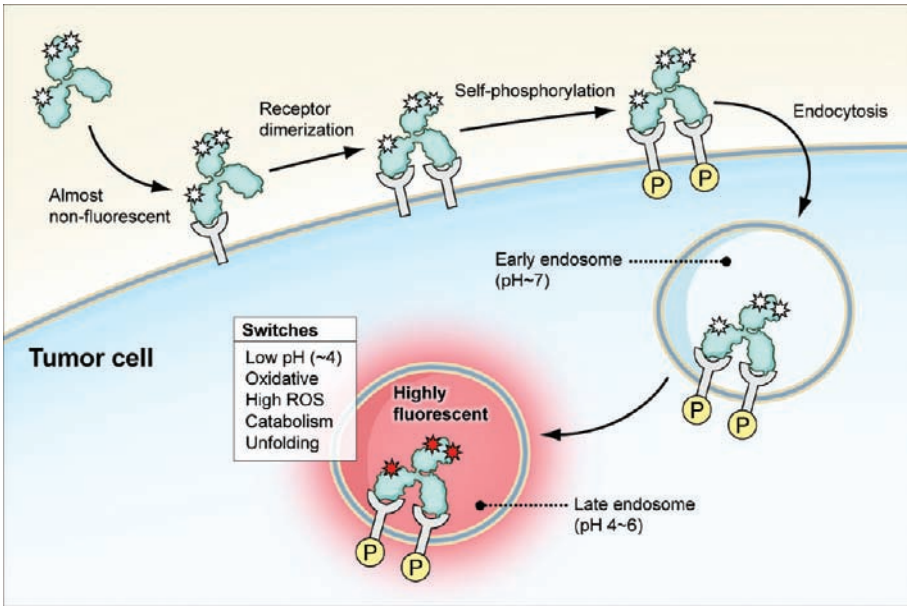

FIGURE 2. Schema for the activation process involving a target-cancer-cell-specific activatable molecular probe based on an antigrowth factor receptor monoclonal antibody conjugated with an activatable fluorophore. Note that the agent is nonfluorescent in the unbound state and becomes irreversibly activated within the target cell.

on the probe. Further, none of the currently utilized enzymes for fluorescence activation are specific for carcinogenesis but are instead released secondary to host−cancer interactions, thereby decreasing specificity. In contrast, probes that are activated by endolysosomal processing are highly specific and generally remain localized to the target, as activation relies upon the probe binding specific cell surface receptors. Signal amplification can be achieved by conjugating multiple fluorophores to a single targeting moiety, such as a monoclonal antibody.[13,14] However, there is a limited number of fluorophores (up to around 10) that can be conjugated to a single molecule without compromising binding specificity, making amplification less potent than that achieved by extracellular enzymatic activation. Due to the highly sensitive (pM range) nature of fluorescence imaging, molecularly targeted cell-specific probes can permit early in vivo cancer detection of extremely small lesions. Moreover, the multitude of cell surface molecules, such as overexpressed growth factor receptors, which are directly related to carcinogenesis, provide numerous targets that are highly specific for cancer. With this in mind, molecularly targeted activation may yield better specificity for detecting cancer than enzymatic activation. In this Account, we focus on the chemical and biological strategies involved in designing target-cell-specific activatable fluorescence imaging probes for in vivo cancer diagnosis.

2. General Strategy for Designing Target-Cell-Specific Activatable Fluorescence Imaging Probes

Targeted molecular imaging probes consist of three basic parts: a targeting moiety for specificity, a carrier to optimize pharmacokinetics, and an activatable fluorophore for signaling. These three components must be integrated in such a way that the biologic specificity, pharmacokinetics, and photochemistry of the probe work in concert to achieve the desired application of the target-cell-specific molecular probe.

2.1. Achieving Biological Specificity. In order to design a target-specific probe, ligands should bind with specificity to the target but not the background tissues. Among the biological targeting molecules, monoclonal antibodies and receptor ligands generally have the highest specificity for their respective antigens or receptors. In addition, large proportions of these ligands are internalized after binding to their targets and then undergo lysosomal processing. Within the lysosome, the antigen−antibody or ligand−receptor pairs are catabolized under unique conditions including low pH, high protease activity, and oxidation, which can separately or collectively serve as a chemical switch to "turn on" the activatable fluorophore. For these reasons, we commonly rely on these cell surface proteins to develop targeting probes (Figure 3).

2.2. Pharmacological Strategy. The pharmacokinetic strategy of target-specific activatable probes is very different

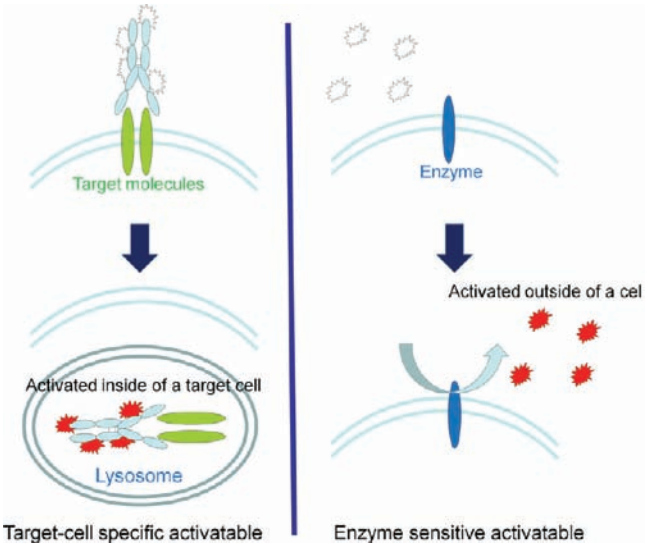

FIGURE 3. Schematic explanation of the function of a target-cancer-cell-specific activatable probe (left) versus an enzyme activatable probe (right). The signal activation of target-cell-specific activatable probes occurs intracellularly, whereas enzyme activation typically occurs in the extracellular environment, permitting diffusion away from the target cell.

from that of conventional "always on" imaging probes.[3] Since target-specific activatable probes do not emit signal before hitting the target molecules, unbound probes do not yield signal. Therefore, there is less background signal to compromise sensitivity and specificity, yielding an absolute increase in target-to-background ratios as the signal from the unbound agent is negligible.[15] Thus, activatable probes can have longer clearance times without compromising imaging, which allows a greater amount of injected reagent to accumulate in target tissue/cells. With "always on" probes, there is a premium on optimizing pharmacokinetics to remove unbound probe as quickly as possible to improve the target-to-background ratio. However, this is not always possible. For instance, monoclonal IgGs labeled with "always on" signaling payloads exhibit prolonged clearance, which leads to high background signal and low target-to-background ratios.[3] Yet, when the same monoclonal IgG is labeled with an activatable signaling payload, high target-to-background ratios can be achieved despite the presence of unbound probe remaining in circulation[14,16] (Figure 1, bottom panel).

2.3. Photochemical Strategy. In order to permit imaging with high target-to-background, the activation ratio (postactivation signal/preactivation signal) must be high. In effect, this means that the signal intensity of the quenched (or off) state of the fluorophore should approach zero. Therefore, optimal dequenchable quenching of the photochemical reaction including H- or J-type dimer formation, homo- or hetro-Förster resonance energy transfer, and photon induced electron transfer (see section 3) is required in the nonactivated state. However, in order to produce an image, the signal generated by activation must have sufficient quantum yields to be detected by a camera. Therefore, an ideal probe yields little or no signal in the unbound state but becomes brightly fluorescent in the activated state after binding and internalization.

3. Photochemical Basis for the Fluorescence Signal Quenching and Activation

Several different photochemical mechanisms for fluorescence signal quenching and activation have recently been discovered. The best known mechanism is Förster (fluorescence) resonance energy transfer (FRET), wherein energy from one fluorophore is transferred to another molecule, when the two molecules are in close (<10 nm) proximity. The FRET pair can consist of two fluorophores (self-quenching) or a fluorophore and a quencher molecule. The FRET or H-dimer formation effect is based on inter-fluorophore processing between two fluorophore molecules in proximity of one another. Another mechanism of activation, which has been more recently discovered, is photon-induced electron transfer (PeT), which operates within a single fluorophore molecule and does not require the presence of a second fluorophore. Both of these mechanisms can then be utilized in the design of target-cell-specific activatable probes.

3.1. Self-Quenching (Homo-FRET). Self-quenching or homo-FRET occurs when excited fluorophores of the same type absorb energy from each other that otherwise would have led to an emitted photon, thus diminishing the fluorescence of the entire compound. This design is commonly employed because of its simplicity. For instance, this mechanism has been employed for producing enzyme activatable imaging probes activated by the cathepsin and matrix metalloproteinase family of enzymes found in cancers and inflammation.[10] However, homo-FRET quenching can only take place when multiple fluorophores are within proximity of one another. Homo-FRET quenching can be validated by the absorbance spectra of the quenched conjugates.[17] While homo-FRET can produce high postactivation signal from the multiple fluorophores involved, there is often considerable preactivation signal reducing the activation ratio (Figure 4a).[11,13,14]

3.2. Quencher−Fluorophore Combination. While self-quenching fluorophores have thus far been limited by

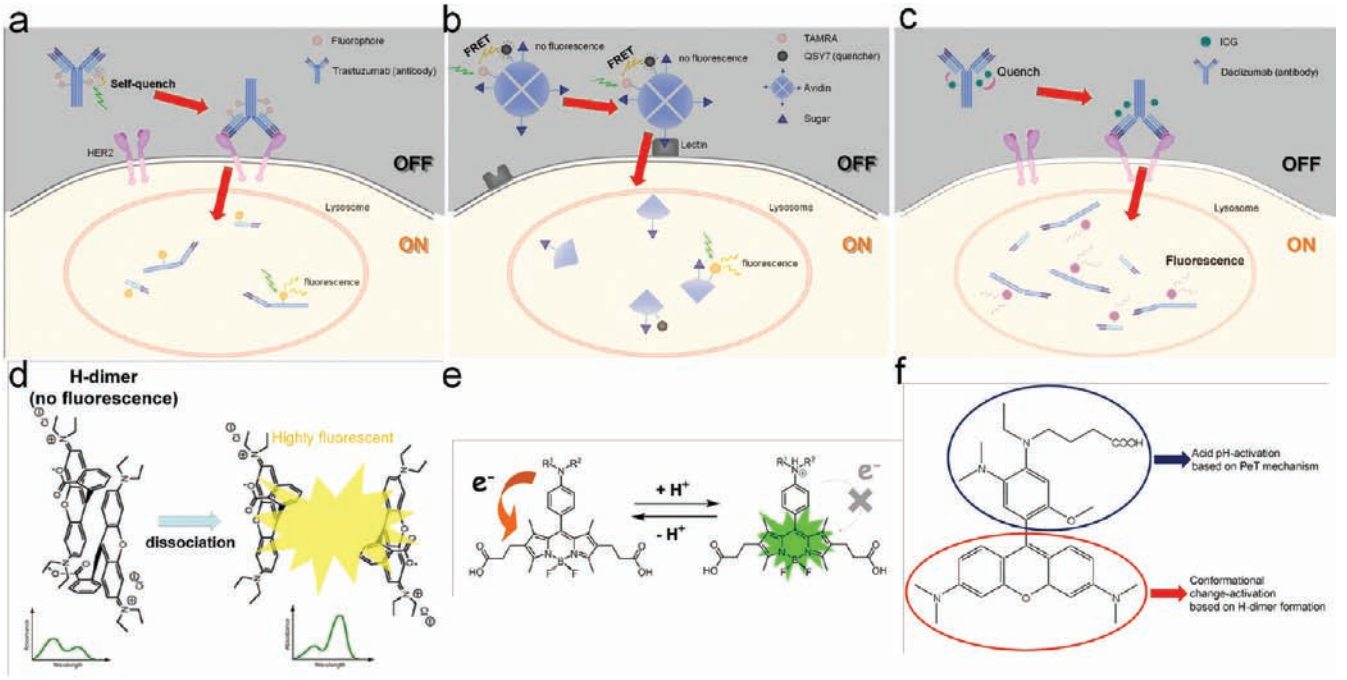

FIGURE 4. Schematic explanation for five available photochemical activation strategies: (a) self-quenching (homo-FRET), (b) quencher−fluorophore combination, (c) autoquenching, (d) H-type dimer formation, (e) photon induced electron transfer (PeT), and (f) a dual functional activatable fluorophore based on the combination of H-type dimer formation and PeT.

background production of fluorescence in the preactivation quenched state, quencher−fluorophore pairs produce little preactivation state fluorescence. Chemical quencher−fluorophore combinations are commonly utilized in the design of small molecular activatable probes. Quencher−fluorophore combinations can achieve higher activation ratios due to low preactivation signal in the quenched state compared to self-quenching activatable probes (Figure 4b).[18] However, this strategy is limited by the intensity of the post-activation signal due to the smaller total number of fluorophores in the probe (compared to the self-quenching strategy, where there are numerous fluorophores available). Furthermore, there are only a limited number of biocompatible quencher−fluorophore pairs with sufficiently high activation ratios.[19]

3.3. Autoquenching. Some fluorochromes can spontaneously induce a low signal quenched state, when conjugated with proteins, likely due to interactions between fluorophores and aromatic rings on the side chain of amino acids, such as tryptophan or phenylalanine. A good example of this is indocyanine green (ICG)-like dye, which can be fully quenched when covalently conjugated with humanized monoclonal antibodies via a side chain of lysine, even at low conjugation ratios (Figure 4c). The activation ratio of ICG-conjugated antibodies can be as high as 50, an extremely high value among the activatable near-infrared probes with emission wavelengths over 800 nm.[16] Moreover, ICG has a long proven record of safety in humans.

3.4. H-Dimer Formation. Xanthene derivatives are known to form homodimers at high concentrations (~mM) in aqueous solutions. This homodimer formation induces short (H-dimer) or long (J-dimer) shifts of absorbance spectra, which completely quench the emission fluorescence signal.[20,21] When covalently conjugated with long proteins, two fluorophores in close (<0.5 nm) proximity can maintain the homo H-dimer formation at a lower concentration (<nM) and yet easily dissociate to dequench after conformational change or unfolding of the protein (Figure 4d). Therefore, H-dimer formation represents an alternative mechanism for designing activatable imaging probes with high activation ratios.[22] Unfortunately, only a few fluorochromes form H-dimers under random protein conjugation reactions. Therefore, even though the fluorochromes forming H-dimers are completely quenched, the remaining fluorochromes in the probe may contribute to preactivation light emission, thus lowering the overall activation ratio of the macromolecular probe.

3.5. Photon-Induced Electron Transfer. Another mechanism for developing activatable fluorescence imaging probes is "photon-induced electron transfer" (PeT). PeT is a widely accepted mechanism for fluorescence quenching, in

which electron transfer from the PeT donor to the excited fluorophore diminishes the fluorescence signal. When the PeT donor is cleaved from the fluorophore or inactivated, full activation is achieved. Recently, it was reported that the fluorescence of the commonly utilized fluorophore fluorescein and its derivatives could, in part, be controlled by the PeT mechanism; that is, the fluorescence properties of fluorescein derivatives could be modulated by intramolecular PeT. However, the PeT mechanism was thought to be limited to lower wavelength fluorophores and was not thought to be possible in longer-wavelength fluorophores. Indeed, almost all the reports of PeT activation strategies utilize UV-excitable fluorophores including anthracene. However, Urano et al. recently found that when the fluorescein structure was deconstructed into two parts, that is, the benzoic acid moiety as the PeT donor and the xanthene ring as the fluorophore, only small reductions in emission were observed with fluorescein and its derivatives. Yet, when the highest occupied molecular orbital (HOMO) energy of the benzoic acid moiety was higher than a certain threshold, PeT occurred much more efficiently, resulting greatly reduced fluorescence.[23] On the other hand, when the HOMO energy is lower than this threshold, as is the case for generic fluorescein, the PeT mechanism is less efficient and the molecule becomes highly fluorescent. This strategy has been used not only with fluorescein but also with a wide range of longer-wavelength families of fluorophores, such as BODIPYs, rhodamines, and even cyanines (Figure 4e). Utilizing the PeT mechanism, fluorochromes can be quenched and activated with extremely high activation ratios, as high as 750.[12] However, the conjugation reactions required for attaching targeting ligands to the PeT fluorophore may affect the HOMO energy state, resulting in a decrease in the effective activation ratio of the entire probe.

3.6. Combination of Different Mechanisms. PeT activation theory relies on *intra-fluorophore* electron donation, whereas the other activation strategies are based on *inter-fluorophore* interactions. Therefore, by employing PeT in combination with interfluorophore strategies, synergic effects may further improve the activation ratio. Ogawa et al. simultaneously employed both PeT and H-dimer formation mechanisms to synthesize an activatable fluorescence probe with higher activation ratios than were achievable with either PeT or H-dimer formation alone. This hybrid agent was able to depict target cancer tissues in vivo with high tumor-to-background ratios during fluorescence endoscopy (Figure 4f).[24]

4. In Vivo Imaging Applications of Target-Cell-Specific Activatable Probes

Signal activation is irreversible in most activatable probes; once activated, these probes will continue to emit fluorescence signal until the fluorophore is physically destroyed or biologically catabolized. Therefore, the signal strength of an irreversible activatable probe is related to the cumulative number of activated molecules in target cells.[11,13,14,16,22] In contrast, signal activation in some activatable probes is reversible; that is, the probe deactivates in response to environmental changes such as pH. Therefore, the signal strength of the reversible activatable probes reflects the number of activated probe molecules at a given moment, whereas the signal derived from irreversible activatable probes is cumulative over time.[12] For this reason, most irreversible activatable probes yield stronger signal and are more sensitive than reversible activatable probes; however, only reversible activatable probes have the potential for real-time monitoring of in vivo events.

4.1. Irreversible Activatable Probes. Target-cell-specific irreversible activatable probes are ideal for highly specific and sensitive cancer detection. The combination of highly specific targeting (e.g., monoclonal antibodies), high activation ratios, and accumulation of the probe in target cells over time enables the detection of cancer nodules as small as $\sim$100 μm in diameter with high (>95%) specificity (Figure 5a).[11,14,16] Since unbound reagent does not contribute to the image, clear images of tiny deposits of cancer can be achieved rapidly with either intravenous or intraperitoneal administration. Moreover, unlike "always on" agents, there is no need to wait for clearance of the injected agent to reduce the background signal, but only the need to wait for activation. The rapidity with which images are formed with this strategy is ideal for numerous real-time clinical applications, including surgical or endoscopic procedures (Figure 5b).[22]

4.2. Reversible Activatable Probes. In addition to target-specific detection, reversibly activatable probes provide the opportunity to monitor the response of cancer cells to therapy in real time. For instance, it is established that lysosomal pH is maintained by an ATP-dependent proton pump. As cells become damaged or die as a result of effective therapy, the activity of ATP decreases, thus reducing the acidification of the lysosome. The activatable pH-sensitive imaging probes within the lysosome then lose fluorescence signal, indicating cellular dysfunction or death. Loss of fluorescence then serves as a surrogate marker for

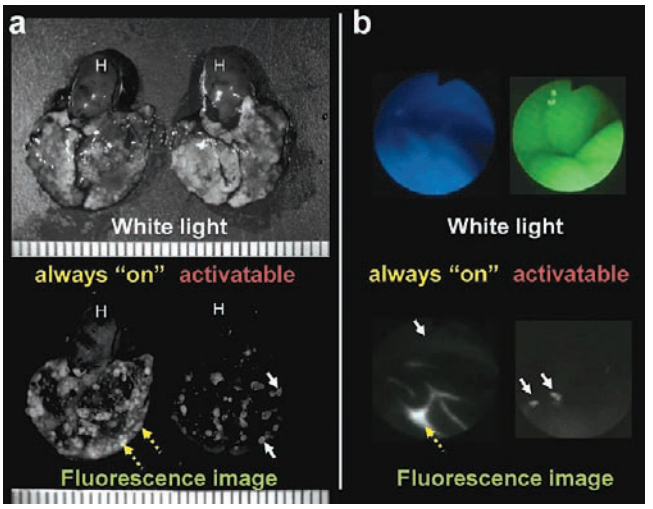

FIGURE 5. Comparison of in vivo imaging utilizing an "always on" probe (right) and a cancer-cell-specific activatable probe (left). (a) Mouse model of metastatic HER2-positive lung cancer imaged 1 day after intravenous injection of "always on" (left) and activatable (right) fluorescence-labeled trastuzumab (monoclonal antibody against the HER2 receptor). The target-cell-specific activatable probe (right) exclusively images targeted lung metastasis (white arrow) without visible background signal remaining unbound reagent within the blood pool. In contrast, the "always on" probe images lung cancer metastases, yet considerable background signal results from unbound agent within the blood vessels (yellow dashed arrow) and heart (H). (b) Mouse model of ovarian cancer with disseminated peritoneal metastases imaged using a fluorescence endoscopy system 2 h after intraperitoneal injection of "always on" (left) or activatable (right) fluorescence-labeled D-galactose lectin binding probe. The target-cell-specific activatable probe (right) only shows targeted peritoneal tumors (white arrow) without background signal from unbound reagent. In contrast, the "always on" probe demonstrates higher signal from unbound reagent (yellow dashed arrow) than that from targeted tumors (white arrow).

successful anticancer therapy. Reversible activatable probes may thereby permit effective real-time monitoring of viable cancer cell burden (Figure 6).[12]

5. Concluding Remarks

Herein, we discuss the chemical, pharmacological, and biological strategies for designing target-cell specific activatable fluorescence imaging probes. We also present a discussion regarding their in vivo application for cancer imaging. Integration of these multidisciplinary approaches enables the rational design and synthesis of target-cell-specific activatable fluorescence imaging probes, which target a variety of different molecules (Figure 7). Such activatable fluorescence probes have demonstrated the potential to depict tiny cancer nodules with high sensitivity and specificity while exhibiting minimal background signal. Successful translation of target-cell-specific activatable imaging to surgical and endoscopic procedures has promise for improving the diagnosis and treatment of cancer.

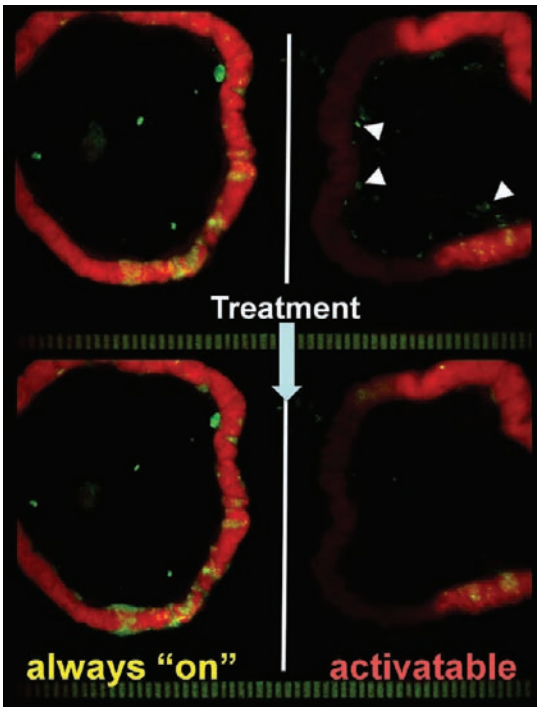

FIGURE 6. Fluorescence imaging depicting real-time therapeutic effects in a peritoneal ovarian cancer model using a reversible pH-sensitive targeted activatable probe. The fluorescence signal derived from the activatable probe decreased with time, indicating successful treatment.

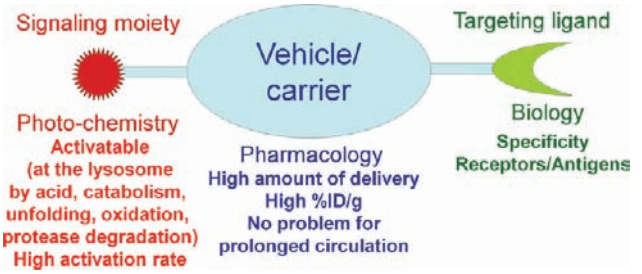

FIGURE 7. Summary of the potential designs for target-cancer-cell-specific activatable fluorescence probes.

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BIOGRAPHICAL INFORMATION

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Peter L. Choyke is the Director of the Molecular Imaging Program at the Center for Cancer Research of the National Cancer Institute in Bethesda, Maryland. Dr. Choyke is a graduate of Jefferson Medical School and received training in Diagnostic Radiology at Yale University and the University of Pennsylvania. He joined the Diagnostic Radiology Department at the Clinical Center of the National Institutes of Health in 1988 and formed the Molecular Imaging Program at NCI in 2004. His interest is in accelerating the treatment of cancer by using novel molecular imaging agents which target specific features of cancers.

FOOTNOTES

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REFERENCES

1 Weissleder, R.; Pittet, M. J. Imaging in the era of molecular oncology. *Nature* **2008**, *452*, 580–589.

2 Alford, R.; Ogawa, M.; Choyke, P. L.; Kobayashi, H. Molecular probes for the in vivo imaging of cancer. *Mol. BioSyst.* **2009**, *5*, 1279–1291.

3 Wu, A. M.; Senter, P. D. Arming antibodies: prospects and challenges for immunoconjugates. *Nat. Biotechnol.* **2005**, *23*, 1137–1146.

4 Louie, A. Y.; Huber, M. M.; Ahrens, E. T.; Rothbacher, U.; Moats, R.; Jacobs, R. E.; Fraser, S. E.; Meade, T. J. In vivo visualization of gene expression using magnetic resonance imaging. *Nat. Biotechnol.* **2000**, *18*, 321–325.

5 Weissleder, R.; Tung, C. H.; Mahmood, U.; Bogdanov, A., Jr. vivo imaging of tumors with protease-activated near-infrared fluorescent probes. *Nat. Biotechnol.* **1999**, *17*, 375–378.

6 Aime, S.; Delli Castelli, D.; Terreno, E. Novel pH-reporter MRI contrast agents. *Angew. Chem., Int. Ed.* **2002**, *41*, 4334–4336.

7 Zhang, S.; Wu, K.; Sherry, A. D. A Novel pH-Sensitive MRI Contrast Agent. *Angew. Chem., Int. Ed.* **1999**, *38*, 3192–3194.

8 Kobayashi, H.; Ogawa, M.; Alford, R.; Choyke, P. L.; Urano, Y. New Strategies for Fluorescent Probe Design in Medical Diagnostic Imaging. *Chem. Rev.* **2010**, *110*, 2620–2640.

9 Mahmood, U.; Tung, C. H.; Bogdanov, A., Jr.; Weissleder, R. Near-infrared optical imaging of protease activity for tumor detection. *Radiology* **1999**, *213*, 866–870.

10 Tung, C. H.; Bredow, S.; Mahmood, U.; Weissleder, R. Preparation of a cathepsin D sensitive near-infrared fluorescence probe for imaging. *Bioconjugate Chem.* **1999**, *10*, 892–896.

11 Hama, Y.; Urano, Y.; Koyama, Y.; Kamiya, M.; Bernardo, M.; Paik, R. S.; Shin, I. S.; Paik, C. H.; Choyke, P. L.; Kobayashi, H. A target cell-specific activatable fluorescence probe for in vivo molecular imaging of cancer based on a self-quenched avidin-rhodamine conjugate. *Cancer Res.* **2007**, *67*, 2791–2799.

12 Urano, Y.; Asanuma, D.; Hama, Y.; Koyama, Y.; Barrett, T.; Kamiya, M.; Nagano, T.; Watanabe, T.; Hasegawa, A.; Choyke, P. L.; Kobayashi, H. Selective molecular imaging of viable cancer cells with pH-activatable fluorescence probes. *Nat. Med.* **2009**, *15*, 104–109.

13 Hama, Y.; Urano, Y.; Koyama, Y.; Gunn, A. J.; Choyke, P. L.; Kobayashi, H. A self-quenched galactosamine-serum albumin-rhodamineX conjugate: a "smart" fluorescent molecular imaging probe synthesized with clinically applicable material for detecting peritoneal ovarian cancer metastases. *Clin. Cancer Res.* **2007**, *13*, 6335–6343.

14 Ogawa, M.; Regino, C. A.; Choyke, P. L.; Kobayashi, H. In vivo target-specific activatable near-infrared optical labeling of humanized monoclonal antibodies. *Mol. Cancer Ther.* **2009**, *8*, 232–239.

15 Weissleder, R. A clearer vision for in vivo imaging. *Nat. Biotechnol.* **2001**, *19*, 316–317.

16 Ogawa, M.; Kosaka, N.; Choyke, P. L.; Kobayashi, H. In vivo molecular imaging of cancer with a quenching near-infrared fluorescent probe using conjugates of monoclonal antibodies and indocyanine green. *Cancer Res.* **2009**, *69*, 1268–1272.

17 Ogawa, M.; Kosaka, N.; Urano, Y.; Choyke, P. L.; Kobayashi, H. Activatable optical imaging probes with various fluorophore-quencher combinations. *SPIE Proc.* **2009**, *7190*, 6449–6418.

18 Ogawa, M.; Kosaka, N.; Longmire, M. R.; Urano, Y.; Choyke, P. L.; Kobayashi, H. Fluorophore-quencher based activatable targeted optical probes for detecting in vivo cancer metastases. *Mol. Pharmaceutics* **2009**, *6*, 386–395.

19 Ogawa, M.; Kosaka, N.; Urano, Y.; Choyke, P. L.; Kobayashi, H. Activatable optical imaging probes with various fluorophore-quencher combinations. *Proc. SPIE* **2009**, *7190*, 71900Z.

20 Packard, B. Z.; Komoriya, A.; Toptygin, D. D.; Ludwig, B. Structural Characteristics of Fluorophores That Form Intramolecular H-Type Dimers in a Protease Substrate. *J. Phys. Chem. B* **1997**, *101*, 5070–5074.

21 Halterman, R. L.; Moore, J. L.; Mannel, L., M. Disrupting aggregation of tethered rhodamine B dyads through inclusion in cucurbit[7]uril. *J. Org. Chem.* **2008**, *73*, 3266–3269.

22 Ogawa, M.; Kosaka, N.; Choyke, P. L.; Kobayashi, H. H-type dimer formation of fluorophores: a mechanism for activatable, in vivo optical molecular imaging. *ACS Chem. Biol.* **2009**, *4*, 535–546.

23 Urano, Y.; Kamiya, M.; Kanda, K.; Ueno, T.; Hirose, K.; Nagano, T. Evolution of fluorescein as a platform for finely tunable fluorescence probes. *J. Am. Chem. Soc.* **2005**, *127*, 4888–4894.

24 Ogawa, M.; Kosaka, N.; Choyke, P. L.; Kobayashi, H. High Sensitivity Detection of Cancer in vivo using a Dual-Controlled Activation Fluorescent Imaging Probe Based on H-dimer Formation and pH Activation. *Mol. BioSyst.* **2010**, *6*, 888–893.