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The Insatiable Quest for Near-Infrared Fluorescent Probes for Molecular Imaging**

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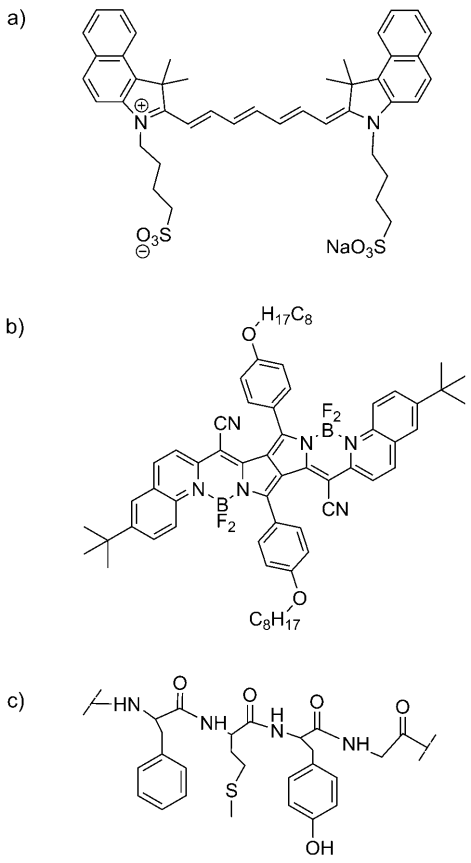
Strategies for the development of new molecular sensors and reporters have captivated the attention of researchers for many centuries. These molecular probes have been instrumental to many pioneering discoveries in chemistry, biology, and medicine. Until recently, most of the available dyes were photoactive at the visible wavelengths, and analytical instruments were optimized for use in this region.

Although visible dyes continue to play important roles in various research endeavors, the advent of optical imaging of molecular processes in living organisms has stimulated interest in the development of molecular probes for use in the near-infrared (NIR) region, typically between 700 and 900 nm. In this spectral window, many intrinsic tissue chromophores, macromolecules, and organelles have low light absorption, autofluorescence, and light scattering. The net effect is that NIR light can penetrate much deeper into tissue than light at the visible wavelengths, thus enabling the assessment of molecular and physiological events in several tissue layers. To harness the advantages of NIR optical molecular imaging, concerted efforts to develop new NIR imaging methods and molecular probes (Scheme 1) have surged in the last decade.

The dye indocyanine green (ICG) naturally became the gold standard for in vivo optical imaging because of its excellent NIR spectral properties and suitability for use in humans. For the investigation of specific molecular processes in vivo, several ICG derivatives have been prepared for subsequent conjugation with peptides, antibodies, and other biologically relevant molecules.[1,2] A major problem with receptor-targeted molecular probes is the occasional lag time between uptake in target tissue and clearance from surrounding tissue. This shortcoming was addressed by the development of activatable NIR probes for in vivo use.[3] Conceptually, activatable NIR probes should only emit fluorescence in response to a specific molecular event. These probes have been used successfully to report the expression of diverse molecular processes. However, earlier activatable probes were based on polymeric materials, which have limited access to intracellular enzymes. There were also concerns about product reproducibility and the slow enzyme-dependent fluorescent enhancement with theses probes. These concerns have led to the development of simpler probes based



Scheme 1. a,b) Chromophores of the NIR-fluorescent carbocyanine dye ICG (a) and diketopyrrolopyrrole cyanine dyes (b); c) chromophore-forming peptide residues of NIR-fluorescent proteins (mNeptune, Katushka, Katushka-9-5, eqFP650, and eqFP670).[4]

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primarily on fluorescence resonance energy transfer (FRET) instead of a self-quenching mechanism. The fluorescence-quenching efficiency of these simple FRET probes is still not optimal, and efforts are under way to optimize the fluorescence quenching and specific activation by enzymes.

Although researchers continue to develop new photostable NIR-fluorescent dyes with high quantum efficiency, highly luminescent quantum dots,[5] and a variety of NIR-fluorescent nanoparticle constructs, an overarching issue in optical molecular imaging is the target specificity of the probes. A new breed of fluorescent and bioluminescent molecular probes excels in this area. These biomolecules have unparalleled specificity because of the seamless incorporation of reporter genes into host cells. The transfected cells are either used directly for cellular imaging or injected into living animals to report the occurrence and dynamics of specific molecular events. Clearly, fluorescent and bioluminescent proteins have different signal-generating mechanisms, but both emit light in the visible region. The realization that NIR spectral signatures are important for noninvasive small-animal imaging has accentuated the need to develop novel proteins that emit fluorescence in the NIR region. Concerted efforts to generate new fluorescent proteins have relied on mutation of the fluorophore in proteins. These efforts recently resulted in the development of NIR-fluorescent proteins with emission at wavelengths longer than 650 nm.[4,6] For bioluminescent proteins, however, signal generation is based on biochemical reactions between an enzyme and its substrate. Hence, the emission wavelength is not dependent on the enzyme chromophore system as with fluorescent proteins. Efforts to shift the emission to longer wavelengths through modification of the substrate have not made much headway, since structure perturbation may disrupt the enzyme–substrate molecular recognition.

The breakthrough for NIR-bioluminescent proteins came with the development of a bioluminescence energy transfer (BRET) method based on quantum dots (QDs).[7] BRET was originally introduced to monitor molecular interactions.[8] Thus, bioluminescence energy is transferred to a fluorescent protein or an organic dye with good absorption spectral overlap but red-shifted fluorescence. However, the small Stokes shift of organic protein fluorophores complicates data analysis because of the need to separate bioluminescence from the resulting fluorescence. In contrast, QDs are ideal for this strategy because they have broad absorption spectra and a large Stokes shift. The availability of several QDs with emission in the NIR region enables researchers to harness the strengths of bioluminescence (highly specific luminescence without the need for external excitation by light) and NIR emission by QDs for in vivo imaging. In this approach, QDs are labeled with a bioluminescent protein (luciferase), and the luciferase substrate can be added to generate BRET. This product was successfully used for cell tracking in rodents after loading the cells with luciferase-linked QDs.[7] The advantage of this approach is that the size and optical properties of the QDs are optimized separately before conjugation with the enzyme.

Ma et al.[9] recently reported a new and elegant method for the synthesis of QDs which takes advantage of the fact that proteins, such as albumin, are used in the preparation of QDs. In this unconventional approach, luciferase was incorporated during the synthesis of the QDs (Figure 1). The

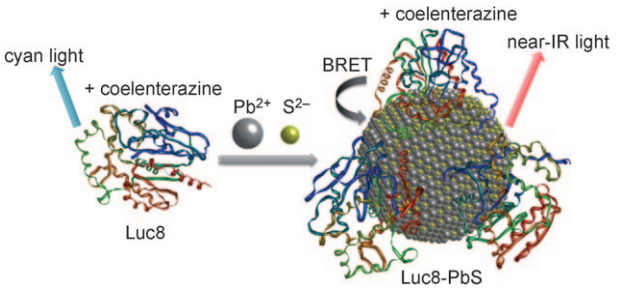

Figure 1. Luciferin-templated synthesis of QDs and BRET. The figure was provided by J. Rao.[9]

enzyme serves a dual purpose. First, it mediates the growth and stability of the QDs, as demonstrated with other, nonluminescent proteins. Second, the luciferase serves as the source of light for BRET. Impressively, the enzyme retained its catalytic properties at the completion of the QD synthesis as a result of the use of Luc8, a more stable mutant of *Renilla reniformis* luciferase. Excellent luminescence in the NIR region was observed with QDs with a mean diameter of 4 nm and a mean hydrodynamic diameter of 20 nm. The small-sized BRET QDs can extravasate to target tissue distal from blood vessels. Importantly, this approach demonstrates the potential of using functionally active biomolecules in nanoparticle synthesis and avoids the need for subsequent conjugation reactions. It also facilitates the in situ generation of the final products with the desired biological functions, stability, and optical properties. Moreover, the strategy provides a unique opportunity to explore nanoparticle preparation with other biomolecules of interest, such as antibodies, diagnostic enzymes, and protein receptors.

An obvious limitation of this method is the need for relatively large amounts of the enzyme for QD synthesis. Thus, the approach may be confined to biomolecules that are available in large quantities and at reasonable cost. Another potential problem is the difficulty of further functionalizing the QDs after enzyme-templated synthesis. Modification of the nanoparticle surface to enable additional functionalization steps may compromise enzyme function. Moreover, some bioactive molecules are highly sensitive to many factors, including the temperature, reactants, and reaction medium needed for the preparation of the nanoparticles. In this case, if more stable mutants are not available, the native protein will lose its biological activity in the final BRET product. For in vivo imaging of molecular processes, additional modification may be needed to make the materials functionally responsive to the biological processes of interest. Unlike fluorescent proteins used for BRET, QDs cannot be genetically encoded into cell DNA. Thus, a major advantage of bioluminescent and fluorescent proteins is lost in this approach. Fortunately, the recent development of an NIR-fluorescent protein may facilitate the design of BRET for NIR fluorescence imaging with genetically encoded molecular systems.

Overall, the method reported by Ma et al.[9] represents a new direction in the preparation of biologically active protein-templated nanoparticles. The study demonstrates that, in the case of luciferase, the biological functions of a biomolecule used as a QD stabilizer can be retained. Without doubt, it opens new opportunities for the design of elegant nanoparticles that incorporate molecular-reporting elements for specific cellular or physiological processes. The search for ideal NIR molecular probes will continue in the foreseeable future. Realistically, no single approach or molecular system will address all the needs of the diverse biological questions awaiting investigation by in vivo imaging. Future developments in NIR molecular probes will be driven by specific biological needs, which will certainly stimulate the ingenuity of chemists, biochemists, materials scientists, and molecular biologists to find solutions to the challenge.

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