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

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Strategies to develop 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 endeavours, the advent of optical imaging of molecular processes in living organisms has stimulated interest in developing molecular probes for use in the near-infrared (NIR) region, typically between 700 nm 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 in tissue relative to the visible wavelengths, 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 (Figure 1) have surged in the last decade.

Naturally, the dye indocyanine green (ICG) became the gold standard for in vivo optical imaging because of its excellent NIR spectral properties and precedence for use in humans. To interrogate 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 developing NIR activatable probes for in vivo use.[3] Conceptually, NIR activatable probes should only emit fluorescence in response to a specific molecular event and the materials have been used successfully to report the expression of diverse molecular processes. However, earlier activatable probes were based on polymeric materials that have limited access to intracellular enzymes. There were also concerns about product reproducibility and slow signal generation needed for optical imaging. These concerns have led to the development of simpler probes based primarily on fluorescence

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resonance energy transfer instead of a self-quenching mechanism. The fluorescence quenching efficiency of these simple FRET probes is still not optimal and efforts are underway 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 used either 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 non-invasive small animal imaging has accentuated the need to develop novel NIR-emitting proteins. 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 >650 nm range.[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 improvement because structure perturbation may disrupt the enzyme-substrate molecular recognition.

The breakthrough for NIR bioluminescent proteins came with the development of quantum dots (QDs)-based bioluminescence energy transfer (BRET) method.[7] BRET was originally introduced to monitor molecular interactions.[8] Here, 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 large Stokes shift. The availability of several QDs with NIR emission allows researchers to harness the strengths of bioluminescence (highly specific luminescence without the need for external excitation light) and NIR ODs for in vivo imaging. To accomplish this goal, QDs are labelled with bioluminescent protein (luciferase) and the substrate luciferase can be added to generate BRET. This product has been 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.

Considering that proteins such as albumin are used in QDs preparation, Ma et al.[9] recently reported a new and elegant QDs synthesis method. This unconventional approach incorporates luciferase in the QDs synthesis (Scheme 1). The enzyme serves a dual purpose. First, it mediates QDs growth and stability, similar to what has been demonstrated with other non-luminescent proteins. Second, the luciferase serves as the source of light for BRET. Impressively, the enzyme retains its catalytic properties at the completion of the QD synthesis. This was made possible because the authors used Luc8, a more stable mutant of *Renilla reniformis* luciferase. Excellent luminescence in the NIR region was observed with 4 nm mean diameter and 20 nm mean hydrodynamic diameter-sized QDs. The small sized BRET-QDs can extravasate to target tissue distal from blood vessels. Importantly, this study demonstrates the potential of using functionally active biomolecules in nanoparticles synthesis and avoids the need for subsequent conjugation chemistries. It also facilitates in situ generation of final products with the desired biological functions, stability, and optical

properties. Moreover, the strategy provides a unique opportunity to explore other biomolecules of interest such as antibodies, diagnostic enzymes, and protein receptors in nanoparticle preparations.

An obvious limitation to this method is the need for relatively large amounts of the enzyme for the preparation. This suggests that 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. This will require modification of the nanoparticle surface for additional functionalization steps, which may compromise enzyme function. Moreover, some bioactive molecules are highly sensitive to many factors, including temperature, reactants, and reaction medium needed to prepare 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 protein-based BRET, QDs cannot be genetically encoded into cell DNA, which loses a major advantage of bioluminescent and fluorescent proteins. Fortunately, the recent development of a NIR fluorescent protein may facilitate the design of BRET for NIR fluorescence imaging using genetically encoded molecular systems.

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

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Figure 1.

Chromophores of NIR fluorescent carbocyanine dye (ICG, **A**), diketopyrrolopyrrole cyanine dye (**B**), and chromophore-forming peptide residues of NIR fluorescent proteins (mNeptune, Katushka, Katushka-9-5, eqFP650, and eqFP670, **C**).[4]

Scheme 1.

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