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Perspectives

Two-photon probes for biomedical imaging

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1. Introduction

Two-photon microscopy (TPM), which employs two near infrared (NIR) photons as the excitation source, has recently become an indispensable tool in biomedical research because of the advantages it offers.[1–5] These advantages include the capability of imaging deep inside live tissues for a long period of time with high spatial resolution. Of particular interest is the ability to obtain hundreds of sectional images along the *z* direction in a thick sample. However, progress in this field is limited because of the lack of efficient TP probes for specific applications. As a result, biologists and clinical scientists are still using commercial fluorescent probes developed for one-photon microscopy (OPM), which have not been optimized for TPM.[6,7] To facilitate the use of TPM in biomedical research, thousands of TP probes are needed for specific applications. In this context, we have initiated a project aimed at developing efficient TP probes. In this Perspective, we will briefly describe the basic principles of TP absorption, model studies, design strategy, specific examples of TP probes developed in our laboratory, and their applications in biology, environment, and medicine.

1.1. Two-photon absorption

The absorption of a photon by a molecule can bring about a single transition to the excited state. Upon irradiation with a high intensity laser beam, however, the same state can be reached by absorbing two photons of lower energy, a phenomenon referred to as two-photon absorption (TPA) (Fig. 1).[8] The possibility of TPA was theoretically predicted by Göppert-Mayer in 1931,[9] and experimentally confirmed by Kaiser and Garrett in 1961.[10]

A lower energy photon has a longer wavelength that can penetrate deeper inside a sample. Since the rate of TP excitation is proportional to δI^2, where δ and I are the TPA cross-section in Göppert-Mayer unit (1 GM=10^{-50} cm^4 s photon^{-1} molecule^{-1}) and the light intensity, respectively,[11–13] and the probability of TPA decreases as a function of $1/r^4$, where r is the distance from the focal point, TP excitation occurs deep inside a sample with intrinsically localized spatial resolution. However, a molecule with a small δ value requires high intensity laser pulses to be TP excited, which can damage the sample. That is why materials with large δ values are needed for practical applications. It is well established that the δ values of the electron donor–acceptor (D–A) dipoles, D–π–D and D–A–D quadrupoles, and two-dimensional octupoles can be increased by increasing the conjugation length and donor–acceptor strength.[11–16] Following this guideline, a large number of efficient TP materials have been developed and successfully utilized in micro-fabrication,[11–13,17,18] three-dimensional optical data storage,[12–14,17–19] optical power limiting,[12,14,20] photodynamic therapy,[13,14,21] and two-photon microscopy (TPM) imaging[3,22–26].

1.2. Model studies on two-photon probes

One of the most promising applications of TPA is bioimaging. In the beginning, there was no precedent literature from which we could learn how to design TP probes. We, therefore, decided to test whether the basic principles developed for the design of one-photon (OP) fluorescent probes such as intramolecular charge transfer (ICT),[27] photo-induced electron transfer (PeT),[28] and resonance electron transfer (RET)[29] might operate in a TP mode. We developed three model TP probes (**1**–**3**, Chart 1), by the synthetic routes shown in Scheme 1.

We first developed **1** as a model TP probe for Ca^{2+} ions.[30] It was derived from 2-cyano-1,4-bis(4-aminostyryl)benzene as the fluorophore and aza-15-crown-5 as the Ca^{2+} ion receptor (Scheme 1). Probe **1** is a turn-off probe with a log *K* value of 4.3 and a 5-fold decrease in the fluorescence intensity in the presence of excess Ca^{2+},

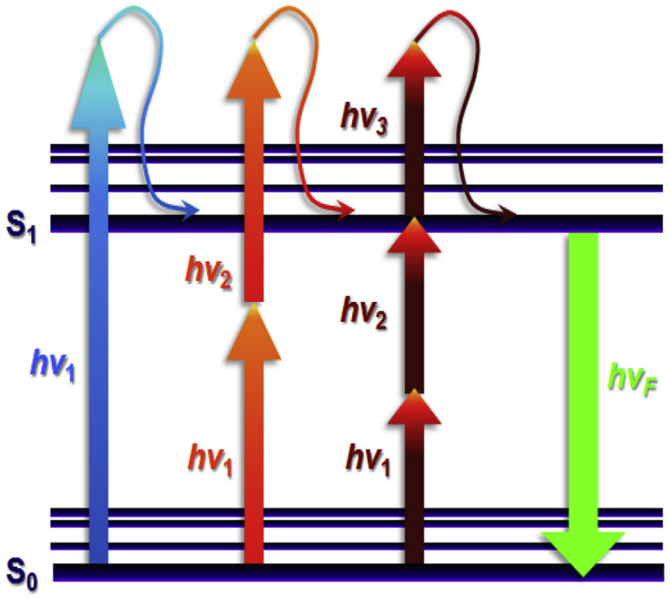

Fig. 1. Energy diagram illustrating one-photon absorption (OPA, blue), two-photon absorption (TPA, orange), and fluorescence (green).

a result that can be attributed to the attenuated ICT from the nitrogen atom in the azacrown ether to the cyanophenyl group during the binding event.[30] Nearly identical results were observed in TP mode, with a log *K* value of 4.3 and 5-fold decrease in the TP excited fluorescence (TPEF) intensity and maximum TP action cross section ($\phi\delta_{max}$) (Table 1). Probe **2**, derived from 2,5-dicyano-1,4-bis(4-aminostyryl)benzene as the fluorophore and bis(2-pyridyl)amine as the metal ion chelator, also showed similar titration behaviors in the OP and TP modes (Table 1).[31] We further developed **3**, derived from 1,3-alternate calix[4]arene having two fluorophores as the pendant groups.[32] In the *apo* state, the TPEF of **3** was negligible. Upon addition of Al^{3+} or Pb^{2+}, the TPEF increased dramatically. This outcome has been attributed to the recovery of the TPEF as a result of the attenuated RET upon metal ion complexation. Here again, the titration behaviors monitored by the OP and TP modes were very similar. These results established that the basic principles underlying the design of OP fluorescent probes can be applied to the design of TP probes.

2. Two-photon probes for bioimaging applications

An efficient TP probe for bioimaging applications should have appreciable water solubility to stain cells and tissues, a significant $\phi\delta_{max}$ value to obtain bright TPM images, high selectivity for the target analyte for selective detection, high photostability, and low cytotoxicity for long-term imaging.[22,23,33] The optimum water solubility is a few μM. It took us almost two years to understand the importance of water solubility for staining. If the water solubility is too high, the probe does not stain the cells probably because it is more stable in aqueous solution than in the intracellular environment.[34] If it is too low, the staining is slowed down and the probe sometimes produces aggregates in the cells that can cause misstargeting problems.[34] The water solubility can be increased by reducing the molecular size of the probe and introducing water-solubilizing groups. To obtain bright TPM images with minimum damage to the samples, the laser power at the objective lens should be less than 5 mW. This requires the probes to exhibit a $\phi\delta_{max}$ value that is greater than 50 GM, which can be achieved by increasing the donor–acceptor strength and conjugation length (Section 1.1). Photostability can be increased by enclosing the conjugation-bridge within the heterocycle, and cytotoxicity can be minimized by reducing the molecular size. The receptors for biological targets