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PHOTOSWITCHABLE NANOFLUOROPHORES FOR INNOVATIVE BIOIMAGING

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Photosensitive fluorescent probes have become powerful tools in chemical biology and molecular biophysics, which are used to investigate cellular processes with high temporal and spatial resolution. Accordingly, photosensitive fluorescent probes, including photoactivatable, photoconvertible, and photoswitchable fluorophores, have been extensively developed during the past decade. The photoswitchable fluorophores have received much attention because they highlight cellular events clearly. This minireview summarizes recent advances of using reversibly photoswitchable fluorophores and their applications in innovative bioimaging. Photoswitchable fluorophores include photoswitchable fluorescent proteins, photoswitchable fluorescent organic molecules (dyes), and photoswitchable fluorescent nanoparticles. Several strategies have been developed to synthesize photoswitchable fluorophores, including engineering combination proteins, chemical synthesis, polymerization, and self-assembly. Here we concentrate on polymer nanoparticles with optically switchable emission properties: either fluorescence on/off or dual-alternating-color fluorescence photoswitching. The essential mechanisms of fluorescence photoswitching enable different types of photoswitchable fluorophores to change emission intensity or wavelength (color) and thus validating the basis of the fluorescence on/off or dual-color photoswitching design. Generally the possible applications of any fluorophores are to label biological targets, followed by specific imaging. The newly developed photoswitchable fluorophores enable super-resolution fluorescence imaging because of their photosensitive emission. Finally, we summarize the important area regarding future research and development on photoswitchable fluorescent nanoparticles.

Keywords: Fluorescent molecular switches; polymer nanoparticles; two-photon; two-photon imaging; spiropyran.

1. Background

The visualization of bioactive molecules and their activities inside living cells is transforming cell biology into a quantitative science. However, despite the brilliant achievements in certain areas of cell biology, most cellular processes and trafficking are still hard to be visualized optically by even current brightest laser excitation. Further advancement will depend not only on improvements in instrumentation but also increasingly on the development of new fluorophores and fluorescent sensors to target these activities. Fluorescent probes are available with a range of colors that span the visible spectrum and a variety of labeling techniques, enabling the simultaneous visualization of multiple targets by multicolor imaging. Furthermore, the revolutionizing development of fluorescent proteins and other genetically encoded fluorescent labels has allowed specific proteins in living cells to be observed in real time.[1]

The biological structures at subcellular level are not available in optical microscopy because of the limited resolution of fluorescence microscopy. Most subcellular structures range from micrometer to nanometer, while the optical resolution is typically limited to 200 nm laterally and 500 nm axially, which limits the resolution in many imaging conditions.[2] Higher-resolution imaging techniques, such as electron microscopy (EM), cannot be used for living cells. To achieve higher image resolutions down to 50−100 nm, which are comparable to EM, fluorescence microscopic techniques with the labeling specificity and live-cell compatibility have been developed to study the nanoscale structure and dynamics of cells and tissues.[3] In this minireview, we will focus primarily on a newly developed concept of photoswitchable nanofluorophores for reversible bioimaging, which are also promising for recently-developed super-resolution imaging.

2. Classification

Photosensitive probes have become powerful tools in chemical biology and molecular biophysics, which are used to investigate cellular processes with high temporal and spatial resolution. However, few synthetic fluorophores suitable for bioimaging have been converted to efficient photosensitive analogues. With limited choices of photoswitchable fluorophores, people manage to develop some novel optical instruments and methods to improve the resolution. Occurrence of each novel photosensitive fluorophores accelerates the advances of imaging techniques and thus biomedical research. Therefore, the members of photoswitchable fluorophores in molecular toolbox must be expanded to match the demands in recent advancements of innovative fluorescence microscopy. To date, photosensitive probes can be classified as three categories: photosensitive fluorescent proteins, photosensitive organic molecules, and photosensitive nanoparticles.

2.1. *Photosensitive fluorescent proteins*

As the small molecular emitter analogues, fluorescent proteins, represented by green fluorescent proteins, have become the critical molecular toolbox in chemical biology. Moreover, new application requirement for super-resolution imaging accelerates the occurrence and high-speed development of photosensitive fluorescent proteins. Photosensitive fluorescent proteins have become important tools in biomedical research as they permit optical highlighting of subpopulations of a targeted protein in living cells. There are three types of photosensitive fluorescent proteins: photoactivatable, photoconvertible, and photoswitchable fluorescent proteins. Photoactivatable fluorescent proteins are those that are transferred from a dark to a bright state with light.[4,5] In contrast, photoconvertible proteins change their emission wavelength upon photostimulation,[6,7] whereas the photoswitchable can be turned on and off reversibly with specific illumination.[8] It would be desirable if a single photosensitive FP would be ideally suited for most, if not all, potential applications.

An ideal photosensitive FPs should possess the great reversibility on tuning either fluorescence ON/OFF or two-color fluorescence. Photoactivation of FPs typically requires a separate excitation source than the one used for imaging (generally with 400-nm irradiation), followed by fluorescent imaging similar to that used for conventional FPs. Ideal photosensitive FPs should be easily photoactivatable/photoconvertible/photoswitchable to generate a high level of contrast.

Despite their local success in biological research, currently certain limitations exist in the chemical biological application of photosensitive FPs. The limited color palette, spectroscopic distinguishability,

dual shift of both excitation and emission wavelengths, the size and oligomeric state, and long period and complicated genetic recombinant process of genetically encoded fluorescent proteins need to be addressed in developing novel photosensitive FPs.

2.2. *Photosensitive organic molecules*

An alternative to generate photosensitive proteins is to approach label proteins specifically with photosensitive synthetic fluorophores (organic molecules).[9] With some optical instruments and methods, some photoswitchable activator−reporter pairs, such as Cy3/Cy5s, could be utilized as photoresponse imaging provided that the limited photosensitive organic fluorophores are in photosensitive toolbox.[10−13] Many photosensitive organic molecules conjugated with emitters can be used as photosensitive fluorophores.[14] They are easily synthesized by chemists and applied in optical information storage and reversible bioimaging. However, we only have the limited candidates of photosensitive fluorophores.[15] The representative photosensitive organic molecules are those that show photochromism, which further results in photoswitchable emission with advanced design of molecular and nanoscale structures.

Since the discovery of the phenomenon of photochromism nearly 150 years ago, photochromism have been investigated as a reversible transformation in a chemical species between two forms having different absorption spectra by photoirradiation.[16] With practical applications of photochromic materials, one of the most important factors is coloring items must have sufficient thermal stability, and the second is the fatigue resistance of photochromic compounds. Currently, photochromic studies have generally focused on the following classes: diarylethenes, azobenzenes, spiropyrans, and spirooxazines and others.[17]

Diarylethenes have since gained widespread interest, largely on account of high thermodynamic stability of the bistable state.[18] They operate by means of a 6-pieletrocyclic reaction (Fig. 1), the thermal analog of which is impossible due to steric hindrance. The materials developed by domestic and foreign researchers are attracting more and more attention.

The photochromic cis-trans isomerization of azobenzenes has been used extensively in molecular switches, often taking advantage of its shape change upon isomerization to produce a supramolecular result (Fig. 2). In particular, azobenzenes incorporated

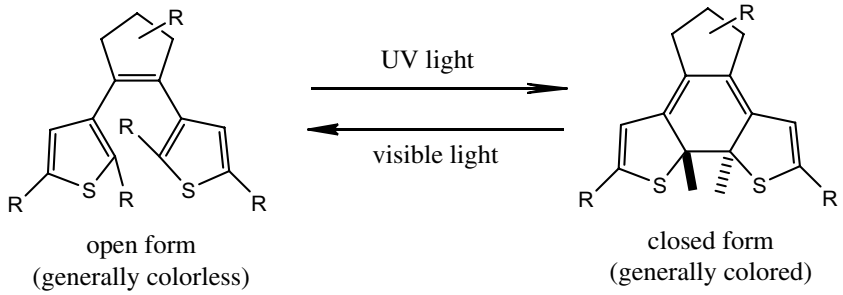

Fig. 1. Photoswitchable mechanism of diarylethenes.

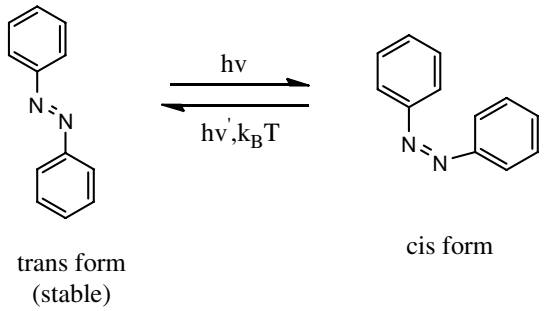

Fig. 2. Reaction mechanism of azobenzenes.

into crown ethers give switchable receptors and azobenzenes in monolayers can provide light-controlled changes in surface properties.[19]

Spiropyrans was one of the most important photochromic compounds. Under near ultraviolet or blue-purple light irradiation ($\lambda_{max} = 300-410$ nm), the C−O bond in spirocycle site will be broken, accompanying the structure transformation of the spiro-carbon atom from sp^3 to sp^2, so planar-form conjugated structure was formed, with the resulting changes from colorless spiropyrans (SPs) to colorful merocyanines (MCs).[20] These photochromics can be used in the field of reversible rewritable storage, in the recent decades, a variety of new applications continue to be found in such fields as optoelectronic devices, ultrahigh-density optical data storage, chemical sensing, and especially for bioimaging. The detailed investigations on SP have produced some new discovery both in theoretical and practical implication.

Fulgides, photochromic Schiff bases, and their heterocyclic compounds are also important photochromic compounds. Fulgide-type photochromic compounds are substituted succinic anhydride derivatives, obtaining extensive studies in recent years.[21] In addition, there are some atypical photochromic compounds, i.e., salicylaldehyde aniline compounds which are photochromic Schiff bases.[22]

2.3. *Photosensitive nanoparticles*

As a new member of photosensitive fluorophore toolbox, photoswitchable nanoparticles have initiated extensive interest from materials and chemistry to biomedical imaging.[23−28]

Photoswitchable polymer nanoparticles containing spiropyrans, originate from photochromism of spiropyrans. Photochromism is a light-induced phenomena, in which chemical species transform between two forms that have distinct absorption spectra.[29] In the past few decades, interest in the photochromic properties of materials has been rapidly expanding.[30−32] Organic molecular photoswitches, especially spiropyrans (SPs), have been widely utilized in the engineering of photochromic materials, including organic polymers and organic−inorganic hybrid thin films.[33−35]

Under NUV irradiation, colorless SPs undergo photoinduced ring-opening reactions, yielding the corresponding isomeric merocyanines (MCs), which absorbs strongly in the visible range of 500−600 nm.[30] Back-conversion from MCs to SPs occurs thermally at relatively slow rates but is significantly accelerated by visible-light illumination.[36]

Previous research interest in SPs focuses mainly on the investigation of the photochromic properties of SPs rather than the fluorescence properties because fluorescence of SPs is rather weak under "normal" conditions.[36] However, the fluorescence is dramatically amplified when SP molecules are incorporated into the hydrophobic nanocavities of polymers.[24] In such environments, alternating NUV- and visible-light exposures cause reversible isomerization between the nonfluorescent spiro- and highly fluorescent mero-forms, turning fluorescence "on" and "off". This is important in optical molecular bioimaging because the fluorescence of the corresponding photochromic nanoparticles can also be optically switched "on" and "off" under irradiation of NUV or visible light. As compared to photochromic signals, which depend on color changes, fluorescence detection is generally more sensitive and thus holds great promise for applications in sensors, biolabels, and bioimaging. For example, the applications of photochromic materials with photoswitchable fluorescence, such as diarylethenes, have resulted in optical information storage, and photoactivatable fluorescent proteins have enabled superresolution imaging of biological samples.[18,37,38]

3. Synthesis and Properties

Spiropyran (SP) is an attractive photochromic material for reversible photoswitching due to the spiropyran−merocyanine (SP−MC) chemistry. SP is referred to as the closed form and is nonpolar, hydrophobic, and colorless under visible light exposure. Photons are an attractive remote external stimuli and can be used to conveniently switch a photochromic materials ON and OFF reversibly. Near UV light (300−410 nm) irradiation of the SP molecule induces a ring opening isomerization that results in the formation of the MC form, which is polar, hydrophilic, and colored. Back conversion to the SP state can be accomplished thermally with relatively slow rates ($t_{1/2} \sim 10^3$ s) but can be accelerated by visible light excitation. Although the fluorescence of SPs is relatively weak under normal conditions, when the SP molecule is confined into hydrophobic solid medium, i.e., the nanocavities of nanoparticles, the fluorescence quantum yield of the MC form is greatly improved (0.24).

The successful synthesis of photoswitchable fluorescent nanoparticles requires incorporating a photochromic molecule into the nanoparticles through covalent or other strong binding. Many strategies to polymer nanoparticles have been applied for photoswitchable nanoparticles.[39−42] Microemulsions are optically transparent isotropic dispersions of water in oil, or oil in water, stabilized with a surfactant and frequently with a co-surfactant. These dispersions are monodispersed spheres with thermodynamic stability and a diameter of less than 100 nm.[23−28] This is in distinct contrast to the properties of macroemulsions, which are optically opaque, kinetically stable, and exhibit diameters within the range of $1-10\,\mu$m. It is well known that many monomers carrying certain polymerizable groups, such as acrylates, methacrylates, and vinyl groups, can be polymerized by emulsion polymerization. Therefore, monomers with different functional groups can be synthesized and covalently linked together in order to modify the microemulsion for specific applications. Combining the attractive confinement and dimensional properties of microemulsions and the chemistry of emulsion polymerization, microemulsions have been successfully exploited in the preparation of photoswitchable fluorescent nanoparticles.

Zhu *et al.*[24] reported the synthesis of polymer nanoparticles by emulsion polymerization that incorporated spiropyran (SP) as the photochromic moiety. The feed materials consisted of initiator 4,4′-azobis(4-cyano-valeric acid), major monomers, N-isopropylacrylamide (NIPAM) and styrene (St), and minor monomers, divinyl benzene (DVB, crosslinker) and the optically active unit, 5-(1, 3-dihydro-3, 3-dimethyl-6-nitrospiro[2H-1-benzopyran-2-2′-(2H)-indole])ethylacrylate (SP) (Fig. 3).

NIPAM is thermosensitive and exhibits a low critical transition temperature at temperatures above 31°C. Therefore, above this temperature the monomer is hydrophobic as the polymerization progresses the resulting poly(N-isopropylacrylamide) (PNIPAM) self-organize into micelles with the aid of a surfactant (Tween). After this initial NIPAM polymerization the hydrophobic monomers (St, DVP, and SP) can participate in the polymerization and be incorporated in the growing polymer chains. Finally the spherical nanoparticles consists of a hydrophobic core and a hydrophilic NIPAM shell. From UV-VIS analysis it was shown that $> 96\%$ of the SP monomer was incorporated into the nanoparticles. The synthesized polymer nanoparticles exhibited ON (UV < 400 nm) — OFF (visible > 450 nm) optical switching behavior due to the photoisomerization between the SP and MC states. Using liposomes, these photoswitchable nanoparticles were delivered into living cells and their presence confirmed by fluorescence imaging.

Using a similar synthetic strategy and methodology, Li and co-workers[26] demonstrated dual color fluorescent nanoparticles for live cell imaging (Fig. 4). Here, two acrylate-containing fluorescent materials, perylene diimide (PDI) and spiropyran (SP), were incorporated into the growing polymer chain and inside the core of the micelle. In this case, the energy of the emitted photon is dependent upon the isomeric state of the photochromic SP molecule. UV irradiation (365 nm) switches the green fluorescence OFF and turns ON the red fluorescence due to the photoisomerization to the MC form. Visible light (488 nm) reverses the fluorescence from red back to green with the back conversion to the SP state. The fluorescence can be manipulated like this due to fluorescence resonance energy transfer (FRET), because the open MC form can quench the green light emitted by the PDI due to its absorption spectrum ($\lambda_{max} \sim 588$ nm) overlapping with the

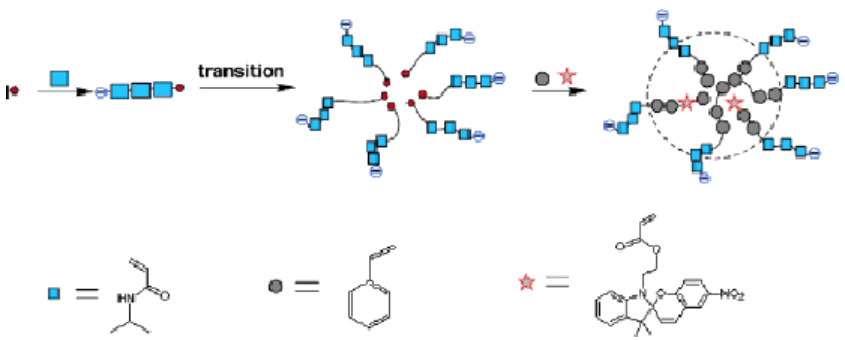

Fig. 3. Schematic representation of the emulsion polymerization procedure.

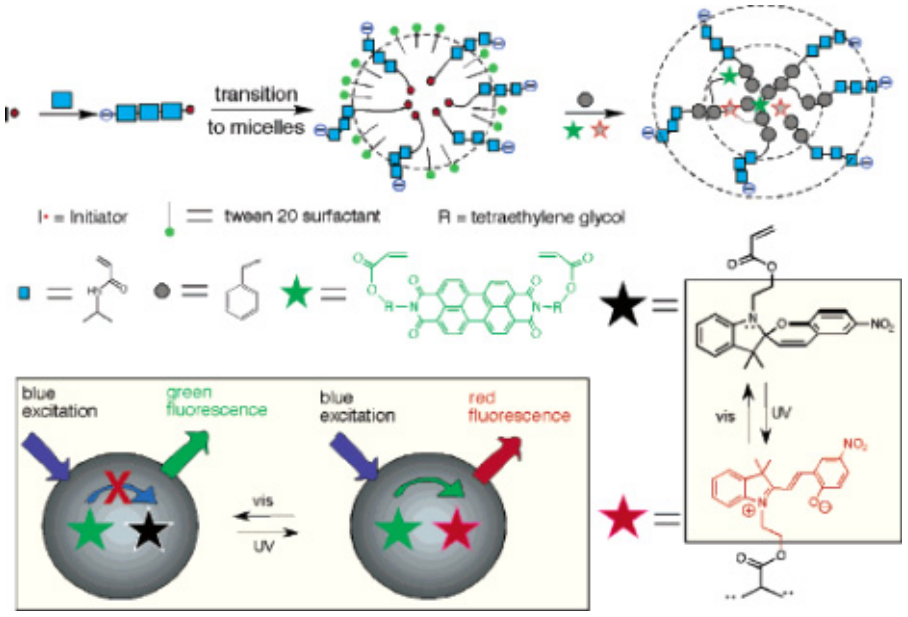

Fig. 4. Schematic representation of the emulsion polymerization procedure.

emission spectrum of PDI ($\lambda_{em} \sim 535$ nm, with overtone at 575 nm). These nanoparticles demonstrated dual-color fluorescence switching in a cytoplasmic cell, and imaging showed the nanoparticles can be selectively highlighted with green or red fluorescence.

All the aforementioned reports utilize one photon absorptions in the visible region as opposed to the near-infrared (NIR) region. Although the NIR is a promising wavelength for bioimaging, there are few fluorophores that absorb in this region. However, two-photon absorption (TPA) using a femtosecond NIR two-photon response allows visible fluorescence possible.[43] Two-photon excitations in the NIR that are lower in energy could thwart the photodamage to biological tissues that may be caused when UV and NUV light is utilized.

Most recently, Zhu *et al.*[23] reported reversible ON-OFF two-photon photoswitching and two-photon fluorescence imaging using SP-based fluorescent nanoparticles synthesized by emulsion polymerization (Fig. 5). Such on-off photoswitching cycles could be duplicated up to 20 times. They demonstrated that immunofunctionalized photoswitchable nanoparticles can be used to target specific membrane receptors such as Her2 on SK-BR-3 cells. The combination of two-photon cross-sections and photoswitchability makes these SP-based nanoparticles an attractive bioimaging agent.

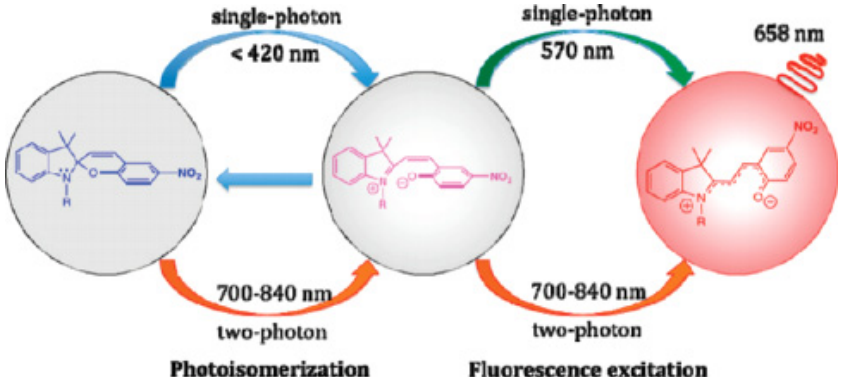

Fig. 5. Single-photon photoswitching and fluorescence excitation contrasted with two-photon photoswitching and fluorescence excitation of SP/MC-containing nanoparticles.

4. Two-Photon Process and Imaging

4.1. *Two-photon photochromism*

The chromophores with a D-π-D, A-π-A or D-π-A (D = electron donor; π = conjugated system; A = electron acceptor) structure have larger two-photon absorbance cross-sections. Belfield and co-workers[44] reported diarylethene dimers having a D-π-D structure in which indole rings are used as electron-donor units and 1,4-bis(ethynyl)benzene or 1,4-bis(ethenyl)benzene as π-conjugation unit. The photochromic reactivity and the TPA cross-sections of the derivatives have been studied.

Figure 6 shows the photoswitch procedures of diarylethene. The color change from yellow to green only occurs at the focus point, under two-photon irradiation for a few seconds with 820 nm light. The green color owes to the photogenerated closed-ring isomer, while the remaining part retained the initial yellow color. This demonstrates that the compound absorbed two photons of 820-nm light and caused the photochromic reaction from the open-ring to the closed-ring isomer. Visible light ($\lambda > 600$ nm) irradiation could erase the green color.

In common photochemistry, photoexcitation occurs through resonant one-photon excitation, which limits the attainable depth and selectivity of photoinduced processes in materials. By comparison, two-photon excitation can have true complete 3D control of the same processes potentially.

Harada *et al.*[45] reported fulgides involves the simultaneous absorption of two photons to produce an excited state normally accessible by absorption of a single photon of twice the energy (half the wavelength) (Fig. 7). The long wavelength light enables deeper penetration of photons into materials. As we know, most fulgides perform their photochromism in the solid state, the photochromic reactions have been hitherto considered to happen on surfaces or at defects of the crystals or to proceed with destruction of the crystals. When irradiating the crystals with steady UV light (405 nm), the incident light could initially penetrate just nearby the surfaces of the crystals, and the internal part of the crystal remained unchanged. Compared to that case, when irradiating the crystals with pulse laser light (742 nm), the colored species distribute much more homogeneously. This means that the

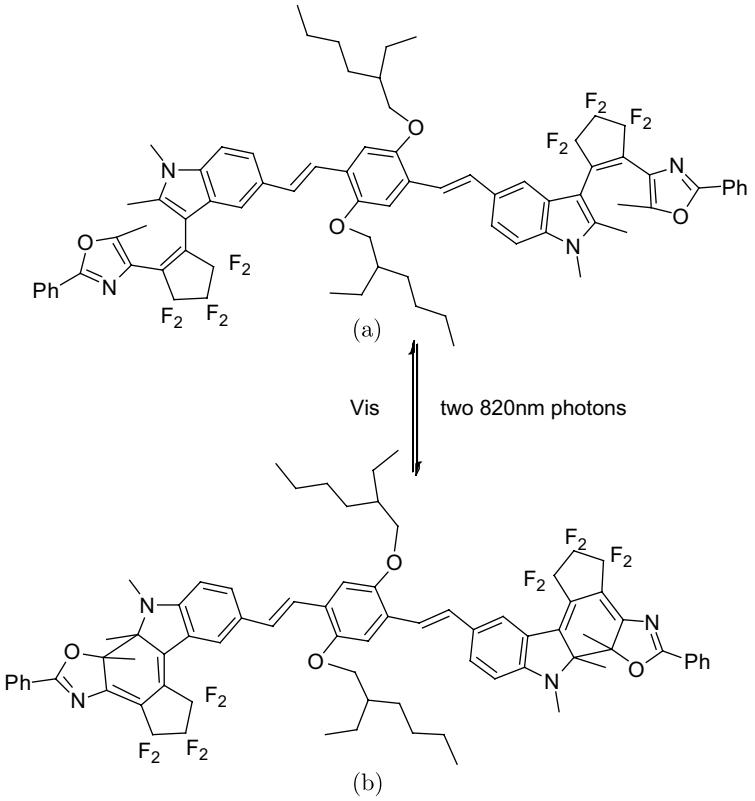

Fig. 6. The photoswitch procedures of diarylethene.

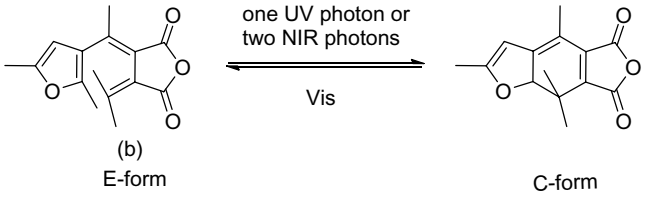

Fig. 7. The photoswitch procedures of fulgide.

photoreaction generated by femtosecond pulsed laser light took place more evenly throughout the crystal.

4.2. *Two-photon fluorescence*

Two-photon-induced fluorescence involves two photons absorbed "synchronously" by the molecule under study to excite an electron in a molecule to an electronic excited state. Subsequently, once in the S_1 state (through internal conversion if a higher excited state was populated), the excited electron relaxes back to the ground state and emits a photon via identical processes as in single-photon absorption and fluorescence processes.[46] This nonlinear process is characterized by the high spatial localization inherent in the quadratic relationship between the excitation and fluorescence intensity.[43] There are many advantages applying two-photon fluorescence in bioimaging, such as promoting penetration depth ($> 500\,\mu$m), fixed target excitation, and spatial resolution, and mitigates tissue autofluorescence, photodamage, and photobleaching.[47]

Compared to the performance of two photons, one-photon excitation cannot offer parallel performance. The application of NIR two-photon fluorescence offers obvious superiority over one-photon fluorescence in areas of 3D optical data storage, two-photon optical power limiting, microfabrication, biological, and medical applications.

4.3. *Two-photon imaging*

As new imaging agents, photoswitchable nanofluorophores possess many potential applications: such as tracking cellular compartments and structures; tracking cells in tissues and embryos; protein diffusion and binding kinetics; and protein−protein interactions. However, the poor axial (z) resolution of conventional, diffraction-limited imaging makes it difficult to spatially confine the photoactivation process in the axial dimension. Two-photon activation is promising to overcome this problem by activating femtoliter to subfemtoliter volumes, providing exclusive control of photoexcitation in nanoscale space. Moreover, since two-photon excitation minimizes light illumination to a great extent in the axial dimension, the risk for phototoxicity in small animal imaging is decreased. These features make two-photon photoactivation a unique tool for many cell and developmental biology applications.

It is well known that both UV- and NUV-light, high in energy, can induce undesired physiological changes in cells, thus causing photodamage to biological tissues.[48] Therefore, circumventing UV-caused photodamage to living systems has motivated many investigators in both optical information storage and bioimaging. To address this problem, two-photon techniques whose wavelengths fall in the NIR optical window, typically between 700 nm−1200 nm, are used.[49] Precisely in this optical window, both the absorption and scattering of unstaining cells and tissues are relatively low, even negligible at certain wavelengths. This, in turn, results in a deeper light-penetrating depth than visible light.[50] Additionally, NIR two-photon excitations will eliminate tissue background from linear fluorescence. In the same context, an additional benefit of two-photon excitation is the reduction in photodamage and hence phototoxicity to living systems outside the focal volume because illumination under two-photon excitations is, in fact, strongly confined to the focal plane.[51]

For most fluorophores, one-photon absorptions occur in the visible spectrum, not in the NIR region. Thus NIR light, albeit a promising energetic wavelength to image biological samples, is frequently not used because of lacking such fluorophores. However, two-photon absorption (TPA) makes visible fluorescence possible using a femtosecond NIR two-photon process.[43] The TPA process involves an electronic transition from the ground state to the excited state by simultaneously absorbing two photons. Such a nonlinear process is named two-photon excitation (TPE), in which the single excited electron returns to its ground state, emitting a higher energetic photon. For example, a femtosecond laser from 700 nm to 1200 nm can excite fluorescent dyes in TPE to impart visible fluorescence. Similarly, TPE can also be used to photoswitch chromophores.

In our recent work, we made use of photochromic and fluorescence properties of spiropyrans (SPs) in the application of bioimaging.[24,26] Alternating irradiations of NUV and visible-light cause reversible

isomerization of SPs between the nonfluorescent spiro- and highly fluorescent mero-forms, switching the fluorescence "on" and "off". The fluorescence of corresponding photochromic nanoparticle in optical molecular bioimaging can also be optically switched "on" and "off" under irradiation with NUV or visible light.

However, UV and NUV light with high energy can lead to undesired physiological changes in cells, thus causing damage to biological tissues.[44] Like organic fluorophores, molecular photoswitches (i.e., MC nanoparticles) do suffer from photobleaching, particularly under UV irradiation. Initially, UV irradiation results in the SP−MC photoisomerization; the increased amount of MC-particles imparts red-fluorescence. Conversely, continuous UV irradiation also causes molecular photobleaching, thus reducing red-fluorescence intensity. In the initial stage, MC population augmentation outperforms MC photobleaching and the red-fluorescence therefore intensifies. In the later stage, fluorescence attenuates as MC photobleaching become predominant and fast-switchable SP population is near depletion. Red-fluorescence intensity maximizes at the equilibrium where positive SP-to-MC photoisomerization and negative MC photobleaching are balanced. This example illustrates that the photobleaching effect on photoswitchable fluorophores remains a challenge for further research.

To solve this problem, we use two-photon techniques whose wavelengths fall in the NIR optical window, usually between 700 nm and 1200 nm. The proposed mechanism of two-photon photochromism is shown in Fig. 8. The photochemical process could

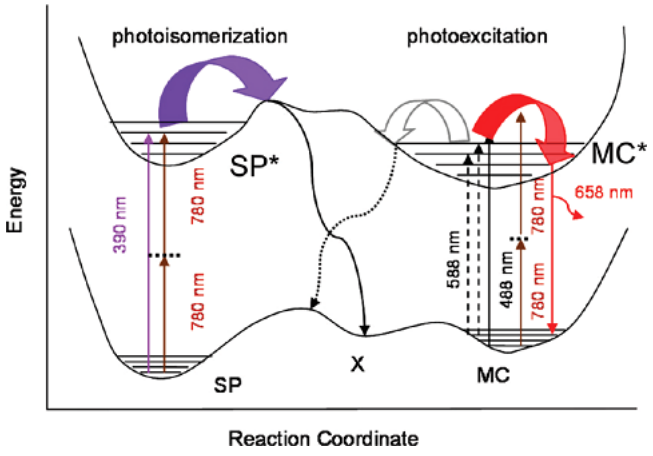

Fig. 8. The proposed diagram illustrating both the emission process and the photochemical process.

be strongly coupled to the fluorescence process, and in this situation, one UV photon (below 415 ± 5 nm) can induce both photoswitching and red-fluorescence, turning from SP to MC induced red-fluorescence. Alternatively, the photochemical process might not be coupled to the fluorescence process. In this case, one UV photon (or two corresponding two NIR photons) would be required to generate photoswitching and another photon near 350 nm (or two corresponding two NIR photons) or 570 nm would be needed to excite the red fluorescence.

For instance, SP nanoparticles, which are usually photoswitched using sub-420 nm light, can also be photoactivated using a sub-840 nm NIR laser in a TPE process (Fig. 8). Under these conditions, TPE not only induced photoisomerization but also imparted red-fluorescence when the SP nanoparticles were exposed to a femtosecond NIR laser (e.g., 780 nm).

Although the detailed mechanism of two-photon NIR-induced SP photoisomerization remains unknown thus far, we have summarized the observed facts in Table 1. Single-photon NUV and two-photon NIR photoswitching produces the same phenomenon — red-fluorescence, indicating that both sub-420 nm light and NIR (700−840 nm) two-photon excitations transform SP-nanoparticles into MC-nanoparticles. SP nanoparticles remain nonfluorescent, but MC nanoparticles emit strong red-fluorescence when excited via either visible single-photon processes or NIR two-photon processes. The salient feature is single-photon fluorescence excitation in the visible region also switch the MC-particles back to the nonfluorescent SP-particles, whereas two-photon excitation does not.

Shown in Fig. 9, A is the bright-field image, B is the fluorescence images of SK-BR-3 cells after incubation with the anti-Her2 antibody-nanoparticle conjugates. Under 780 nm two-photon irradiation, as the photoswitchable nanoparticles had been immunofunctionalized with the targeting anti-Her2 antibody, the membrane Her2 receptors on the SK-BR-3 cells were highlighted with red-fluorescence. To the contrary, MC-particles were turned back to SP-particles under single-photon laser (488 nm) excitations and the red-fluorescence (Fig. 9(c)) was switched off. It was available to demonstrate that the red-fluorescence could be reversibly switched between the two-photon "on" state and the one-photon "off" state, using immunofunctionalized particles and targeted SK-BR-3 cells as the model system. The

Table 1. Effects of various excitation wavelengths on SP- and MC-nanoparticles.

Excitation wavelength (nm)	300−420 nm (one-photon)	420−600 nm (one-photon)	700−840 nm (two-photon)
SP	SP → MC, FL: 0 → high	SP, No FL	SP → MC, FL: 0 → high
MC	MC, FL: high → 0	MC → SP, FL: high → 0	MC, FL: steady
SP + MC	SP → MC, MC: FL	MC → SP, FL: high → 0	SP → MC, MC: FL

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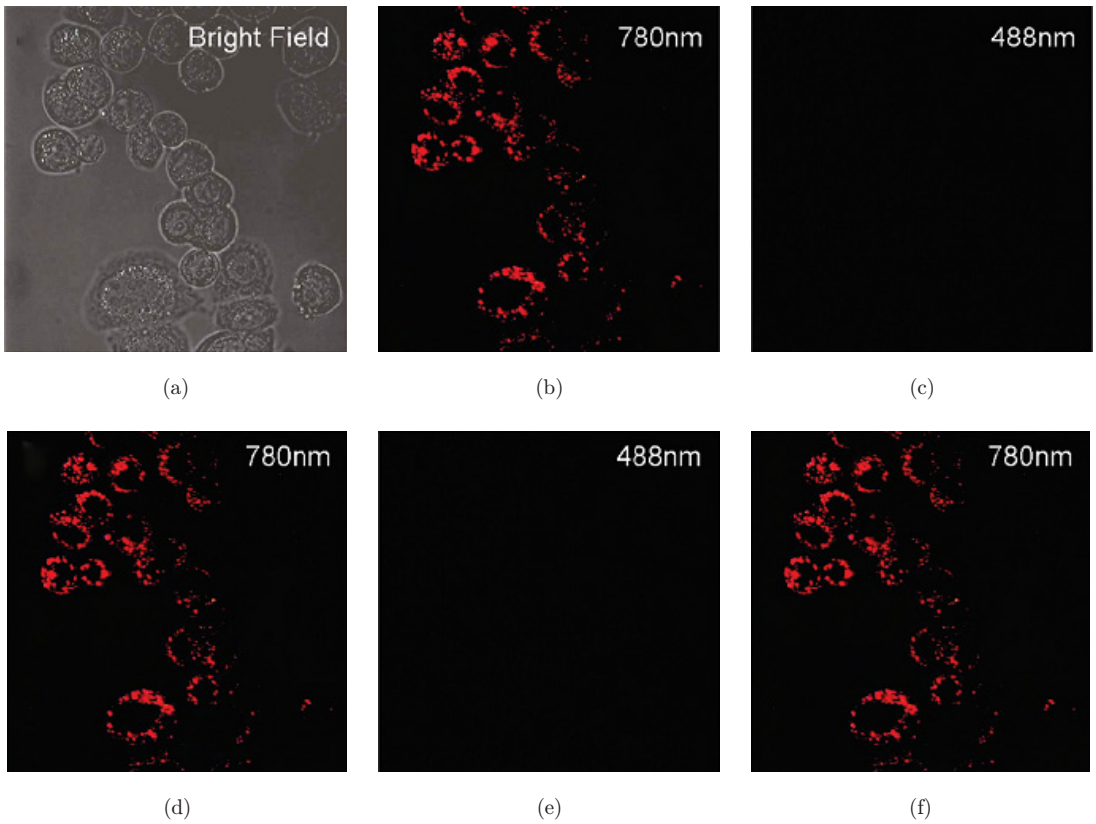

Fig. 9. Alternating NIR two-photon and visible single-photon excitations causes photoswitching of the fluorescence imaging of SK-BR-3 cells labeled by anti-Her2 antibody-conjugated photoswitchable nanoparticles. (a) Bright-field images showing the cell locations. (b) Two-photon photoswitching and two-photon imaging at 780 nm of Her2 receptors on the cellular membranes. (c) Single-photon excitation at 488 nm erases the red-fluorescence image. (d, f) Repeats of (b). (e) Repeat of (c). Such reversible on-off fluorescence imaging of targeted cells could be repeated at least 20 times. Image sizes 146 μm × 146 μm. Reprinted with permission from Ref. 23. Copyright (2008) American Chemical Society.

process of such on-and-off cycles regulated by NIR and visible laser illumination could be circulated at least 20 times (Figs. 9(b)−9(f)). In other words, two-photon excitation at 780 nm gave red-fluorescence, while it was erased by one-photon excitation at 488 nm. Therefore, NIR two-photon photoswitching is a promising alternative to UV irradiation in bioimaging applications using photoactivatable and photoswitchable probes because the issues associated with UV photodamage, phototoxicity, and photobleaching can potentially be alleviated.

5. Perspective

Photoswitchable fluorophores have been focused worldwide since their emergence due to their significance in both fundamental and technological fields. Actually, photoswitchable materials originate from their promising applications in diverse multidisciplinary fields, such as ultrahigh-density optical data storage, optoelectronic devices, chemical and biological sensing and imaging, particularly emerging high-resolution fluorescence microscopic techniques to image cellular samples.[13,14,52−59] Fluorescence

microscopy allows minimally invasive imaging of dynamic processes in live cells in spite of higher resolution but fixed imaging from transmission electron microscopy. Conventional fluorescence microscopy is limited in its spatial resolution, about 200 nm, by the diffraction properties of light. Developing photoswitchable probes are promising to overcome the diffraction limit in far-field fluorescence microscopy and therefore revolutionize the optical imaging field.[60,61] Remarkably, spatial and temporal modulation between two discrete molecular states allows high-resolution optical imaging beyond the diffraction limit. The two exchanging molecular states correspond either to bright and dark fluorescence. Single-molecule fluorophores as imaging agents have unpredictable blinking behaviors, a low photobleaching threshold, and limited brightness. In these regards, assembly of photoswitchable fluorophores could potentially surpass single-molecule fluorophores, because the former usually contains numerous quantum emitters and consequently resolves the photobleaching and photoblinking problems to a great extent.[62,63] Because of their desired properties of high brightness, nonblinking characteristics, and prolonged photobleaching lifetimes, photoswitchable fluorophores have attracted extensive attention. Such enabling nanoparticles in different imaging methods, such as two-photon excitation, and spatial and temporal resolved fluorescence imaging, should greatly contribute to emerging high-resolution fluorescence bioimaging.

Apart from for the two-photon excitation fluorescence bioimaging demonstrated above, another useful application is that photoswitchable fluorophores play revolutionary roles in new super-resolution imaging techniques. Recently, development of super-resolution imaging techniques in far-field fluorescence microscopy has enabled visualizing cellular mechanism at the nanoscale, far beyond the diffraction limit. Current sub-diffraction fluorescence microscopic techniques are based on photoswitching: either "ON" and "OFF" or between two distinct colors. Recently, polymer nanoparticles containing photoswitchable dyes have been used to improve imaging resolution in far-field fluorescence microscopy.[28] A photoactuated unimolecular logical switching attained reconstruction (PULSAR) microscopy was applied to visualize cellular organelles in fixed cells. Promisingly, similar combination of novel imaging agents and newly developed microscopy led to cellular imaging resolution down to 10−40 nm, far beyond the diffraction barrier that conventional microscopy usually encounters. All these new techniques will help to higher-resolution imaging and induce the development of new photoswitchable fluorophores.

Herein, we have highlighted recent advances in photoswitchable fluorophores and their promising applications in biological labeling and imaging. Current photoswitchable probes are still immature, and there is abundant space to decorate their performance before they reach their full potential in practical biomedical and clinical diagnostic applications. However, the results presented so far have already demonstrated that photoswitchable fluorescent nanoparticles are promising in two-photon excitation fluorescent labeling and super-resolution imaging. More efforts are taken to further improve the quality and diversity of photoswitchable nanoparticles. Specifically, smaller in size, brighter in intensity, more resistance to photobleaching, and more photosensitive photoswitchable nanoprobes that emit in the preferred region such as near-IR are expected in the super-resolution imaging.

Obviously, the nanoprobes need to be smaller than the nanoscope resolution. Also, smaller probes minimize perturbation to the biological targets. Such smaller nanoparticles are more suitable for labeling small targets such as subcellular components or a particular protein in an assembly. Thus, new fabrication strategies that can generate smaller photoswitchable nanoparticles with excellent photostability are critically needed. The brightness of individual photoswitchable nanoprobes is very important because the total number of photons that one fluorophore can emit before photobleaching eventually determines the spatial resolution of fluorescence imaging. In addition, the biocompatibility of fluorophores also deserves special attention in the biomedical imaging. Fortunately, we have been in the correct track to hunt appropriate candidates of photoswitchable fluorophores for super-resolution imaging. Several series of photoswitchable fluorophores have been designed and synthesized in our lab. Their optical properties are being investigated. Without doubt, fluorescence photoswitching will bring a brighter future in super-resolution imaging.

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