Photoswitching Nanoparticles Enable Innovative Bioimaging Technologies

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ABSTRACT

Nanoparticles garnished with unprecedented power have evolved to carry multi-technologies in molecular imaging. When a single fluorescent probe is used in bioimaging, it is usually lost in the ocean of myriad interferences and noises generated by the matrix. However, photoswitchable nanoparticles produce oscillating signals that can be selectively amplified to self-identify themselves from interfering noises. Here we show that photoswitchable nanoparticles containing photoactive spiropyran-merocyanine could be imaged not only in time domain (like fluorescent probes), but also in frequency domain (unlike fluorescent probes). Applying frequency-domain imaging (FDI) to biological applications, we have clearly detected, monitored, and tracked photoswitchable probes in living cells over the matrix of overwhelming non-switching fluorescence interferences. Therefore, FDI, a powerful tool, reveals molecular behavior that is not visible in real-time imaging.

Keywords: Nanoparticle, photoswitch, fluorescence, spiropyran, frequency-domain

1 INTRODUCTION

Ultrasensitive and non-invasive, fluorescence imaging has become the method of choice to monitor molecular dynamic in living cells. However, there are several real-world challenges that prevent fluorescence imaging to produce highly reliable information. One such barrier is interfering fluorophors that are randomly and ubiquitously present in most biological samples [1]. As a result, both detection limit and precision suffer.

It is a daunting task for a single molecule's signal to stand out the matrix flooded with interferences and noises. Nanoparticles can integrate multiple technologies to overcome such critical issues [2]. In this report, we will introduce strategies to overcome the problem that a single fluorescent probe is usually lost in the ocean of myriad other molecules in a living cell or unambiguously measuring its signal becomes a daunting task because signals or noises have very little difference in term of characteristic features. Here, fluorescence technology and photoswitching technology are incorporated into a single photoswitchable nanoparticle. When such nanoparticles are under photoswitching excitation, oscillating signals come out and these signals can be selectively amplified in an unlimited manner, thus regaining the critically needed sensitivity, precision, and reliability [3]. Not only such photoswitchable nanoparticles can impart frequency-domain imaging, they can also enable super-resolution imaging of nanoparticles in live cells [4].

Specifically, we integrate a photoswitchable spiropyran and a high-quantum-yield fluorophore into the core of polymer nanoparticles. The photoactive spiropyran undergoes ring-opening reaction to form red-fluorescent merocyanine; this mechanism not only turns on red-fluorescence, but also turns off green fluorescence by resonance energy transfer. Such a periodically oscillating intensities of red- and green-fluorescence impart new technologies to develop frequency-domain imaging (FDI) for biological applications. Using such innovative technologies, one can clearly detect, monitor, and track photoswitchable nanoparticles in complex matrix such as living cells over extended period of time. The significance of such technological integration in nanoparticles imparts what were not visible can now be accurately measured.

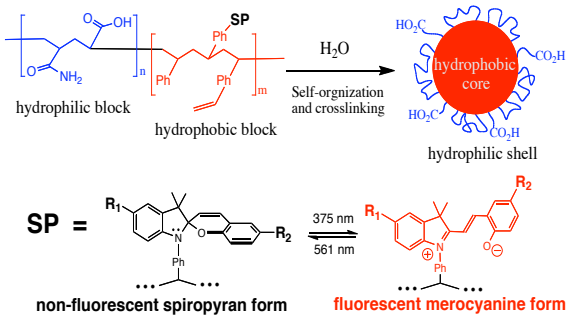

Figure 1: Core-shell nanoparticles were constructed using emulsion polymerization. Hydrophilic monomers such as acrylic acid, acrylamide form the water solvated shell while hydrophobic monomers like styrene, divinyl benzene, and spiropyran functionalized styrene self-organizes into the functional core, resulting in a photoswitchable nanoparticle.

2 RESULTS AND DISCUSSIONS

Figure 1 outlines the strategy of design and synthesis of photoswitchable nanoparticles. While water-soluble monomers are used to form the nanoparticle shells, hydrophobic monomers along with photoswitchable spiropyran functionalized monomers are used to form the cores of the nanoparticles. As a result, the nanoparticles are easily dispersed in water and have functional groups on

surfaces for bioconjugation to proteins/antibodies for specific targeting.

There are a number of strategies to construct photoswitchable nanoparticles. The simplest approach is single color photoswitchable nanoparticles [5]. In this case, the polymerization strategy described in Figure 1 using most spiropyran derivatives will very like produce the desirable red-fluorescence photoswitchable nanoparticles. Alternatively one can integrate a photoswitchable quencher [6] and a fluorescence donor to achieve the same effect [7].

Next generation of photoswitchable nanoparticles is alternating dual-color fluorescence probes; these nanoparticles emit two distinct fluorescence colors, but only one color at a time. In other words, the intensities of these two colors have an anti-phase relationship. One strategy to produce dual-color photoswitchable nanoparticles is to place a fluorescent donor within the Forster distance of the spiropyran photoswitch. When the spiropyran is photoswitched to its counterpart—merocyanine, then fluorescence resonance energy transfer (FRET) will occur from the donor to the merocyanine acceptor. The net results will quench the donor fluorescence, typically green color, and switch on a new color, red-fluorescence in an anti-phase manner [8].

Alternatively, one can construct a molecular photoswitch that simply shuttles between two fluorescence states: the extended conjugate form emitting red-fluorescence, the localized form emitting green fluorescence. Such a design has very high stringent requirements because the molecule must simultaneously have three unique properties. First, the molecule must be a photoswitch, i.e., it responds to an external photo-stimulation and undergoes a specific structural transformation. Second, the localized form must have reasonable quantum yield for high-energy emission, such as blue or green fluorescence. Third, the extended conjugate form must also have decent quantum yield for low-energy emission, like orange or red fluorescence. Even though such demanding requirements are difficult to integrate into a single molecule, we recently successfully designed such a dual-color photoswitch in methoxy-modified spiropyran [9] (Figure 2).

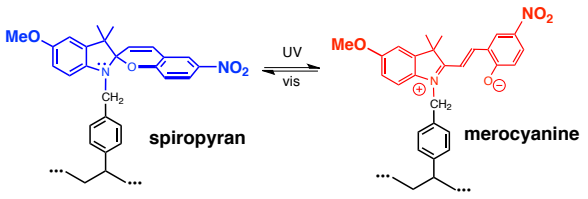

Figure 2: Methoxy spiropyran emits green color fluorescence in the ring-closed state. After a blue-UV light pulse, the methoxy spiropyran will be switched to merocyanine that fluoresces red-color.

After delivering such nanoparticles into live cells, their movements can be tracked by monitoring the periodically bright-and-dark shining dots. As mentioned above, live cells are complicated and they make various molecules and some are fluorescent, thus causing persistent interference. As shown in Figure 3 (left), the interference can basically flood the cellular image and detail movement of the probes as well as the boundary of the cell are difficult to discern. In the time-domain, the probe brightness has reached its ceiling and no viable method can make a single molecule much more brighter. In Figure 3 (top left), there are many photoswitchable nanoparticles near the nuclear of the cell as well as along the cellular membrane. However, their locations cannot be determined as the interferences (yellow patterns and red background) are too strong to resolve the probes. This problem cannot be solved completely in time domain because interfering molecules can be as bright as the sample molecules.

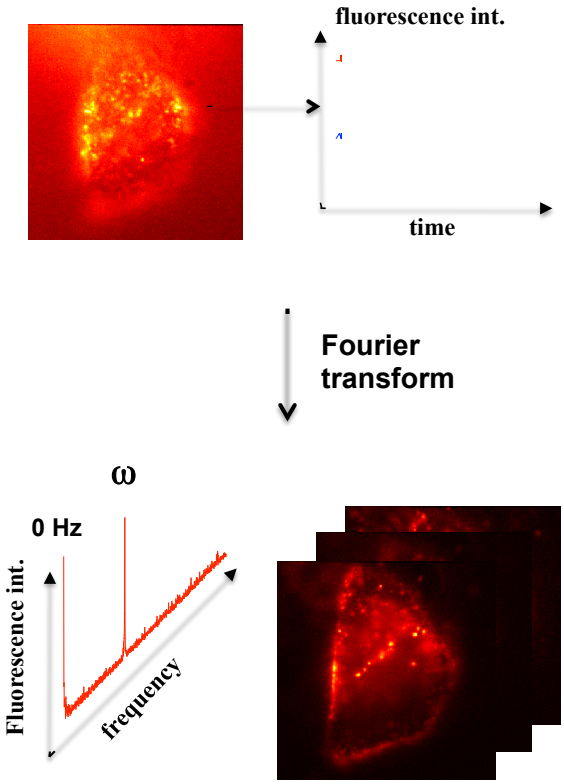

Figure 3: Live cell imaging integrates fluorescence intensity overtime, which reveals considerable interference and background because the boundaries between the living cell and culture medium are not well resolved. However, the fluorescence intensity of photoswitchable nanoparticles oscillates periodically according to the applied photoswitching frequency (ω). Fourier transform identifies this oscillation frequency in the spectrum and oscillating fluorescence intensity in the frequency-domain imaging, which is much sharper than the time-domain image.

Photoswitchable nanoparticles have unique advantages because their fluorescence intensity can be separated from

noises according to frequency (Figure 3, top right). Current fluorophores or nanoparticles do not have unique emission frequency, thus all their intensities will appear at the 0-Hz in the frequency domain (Figure 3, bottom left). In contrast, photoswitchable nanoparticles will appear at the characteristic photoswitching frequency ω, as shown in Figure 3 bottom left. After applying Fourier transform to remove the noise, we obtained a clean copy of the cell image (Figure 3 bottom right), in which cell boundaries are clearly established, organelles inside cell are resolved, and moving spots are sharply identified. Of particular significance, the interfering noises are removed.

For example, those photoswitchable nanoparticles just entered the cellular membrane are clear resolved as a smooth line on the left edge of the cell. The area occupied by the nuclear is dark, indicating that the photoswitchable nanoparticles did not enter the nuclear. Strong interferences away from the dark nuclear region are removed by the Fourier filter and fine features of individual nanoparticles become visible, revealing nanoparticles at various stages from penetrating the membrane to transportation near the nuclear. Specifically, a ridge of nanoparticles that was not visible in the time-domain imaging is clearly resolved in frequency domain imaging. The data shown suggest that frequency-domain imaging of photoswitchable nanoparticles can easily improve the signal-to-noise ratio by more than ten times over that same results from time-domain imaging.

3 EXPERIMENTAL SECTION

Synthesis and characterization of photoswitchable spiropyran dyes have been published previously [8, 9].

The synthesis and characterization of photoswitchable nanoparticles were carried out using emulsion polymerization. When necessary, the monomers were distilled to remove polymerization inhibitors. The resulting photoswitchable nanoparticles were characterized using light scattering and TEM, which revealed that the typical sizes of such particles are 60-100 nm in diameter.

Cell imaging was carried out in a custom-built microscope (Zeiss Axiovert 200 or Olympus X81). The Zeiss Axiovert was equipped with an X-Y nanopositioner for finding the precise location of a single molecule and a liquid-nitrogen cooled CCD for ultra sensitive imaging. The CCD can be switched to a spectrum mode when used in conjunction with a spectrometer (Acton Research Corp). The Olympus microscope was equipped with an ANDOR ion EMCCD for wide field imaging of live cells. Photoswitching was achieved by programming the laser excitation time and synchronizing the EMCCD detection with the imaging laser. For spiropyran-merocyanine pair, the typical on-switching laser is 405 nm, which either turns on only one color, the red-fluorescence or switches green-fluorescence to red fluorescence. Conversely, a 561-nm laser will switch the red-fluorescence back to the green fluorescence or the dark state. The imaging laser is the 488-nm, which fires in synchronization with the EMCCD.

For the dual-color photoswitchable nanoparticles, the red channel uses a long-pass filter (> 620 nm) and the green channel uses a band pass filter (490-550 nm). Typically, the 365-nm laser pulse photoswitches on merocyanine followed by 488-nm fluorescence imaging laser to impart red-fluorescence. This completes a half cycle. The other half cycle starts with a 561-nm photoswitching pulse, which photochemically converts the merocyanine back to methoxyl spiropyran that emits green fluorescence. Once again, the 488-nm fluorescence-imaging laser and the EMCCD come on and collect the data of green-fluorescence. At this stage, a whole switching cycle is complete. These photoswitching pulses, excitation laser, and EMCCD imaging repeat such a cycle again and again to produce the desired oscillation in time domain.

Data processing uses Fourier transform to covert time-domain data into frequency-domain images as shown in Figure 3.

4 CONLUTIONS

Photoswitching nanoparticles, along with photoswitching single molecules can revolutionize fluorescence imaging. A key-enabling factor to these new technologies is the photoswitchable fluorescent fluorophors. As various fluorescence-imaging methods continue to evolve, new molecular probes with optimized chemical and physical properties will be important for developing new imaging capabilities. The average total number of photons emitted per switching cycle scales with the absorptivity and quantum yield and represents an important parameter for performance. The chemistry for developing new photoswitchable molecules with high ultra high brightness and well-behaved photoswitching behavior remains as a high priority in probe development. High photon counts per switching will lead to high on-to-off switching ratio, larger amplitude in real time oscillation; FDI peak brightness increases linearly with oscillation amplitude. Thus, the higher the photon counts per switching cycle, the brighter the FDI peak intensity.

Another important direction is to develop smaller photoswitchable nanoparticles or single photoswitchable fluorophores. As the physical size and weight of the probe becomes important, compact and bright photoswitchable nanoparticles or a single molecule with all the desired properties will have significant impact in bioimaging.

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