Imaging Agents

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Live-Cell-Permeable Poly(*p*-phenylene ethynylene)**

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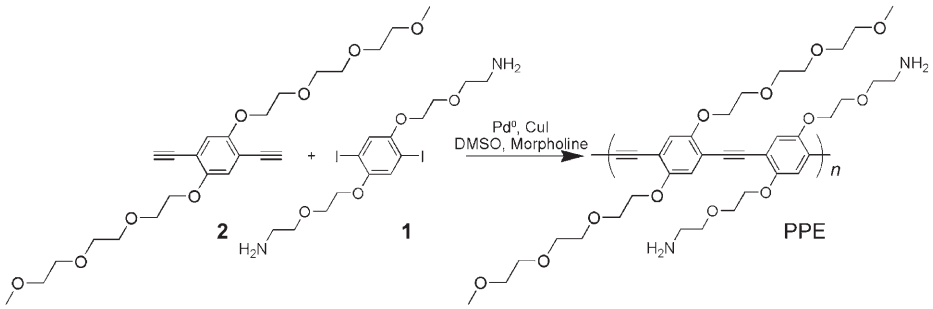
Fluorescent labeling and detection of target biological molecules in live cells is an essential way of studying complex and dynamic cellular processes.[1] Many fluorescent dyes[2] and engineered fluorescent proteins[3] are widely used for these applications because of their small size and biocompatibility. However, poor photostability of these probes limits their broad applicability in long-term monitoring of live cells with high sensitivity. Quantum dots (QDs) are considered an alternative probe owing to their excellent optical properties, such as high photostability, narrow emission, and high brightness.[4] However, the inherent toxicity of QDs (mainly from a heavy-metal core, such as divalent cadmium ions) causes concern in long-term monitoring of cellular events.[5,6] In addition, difficulties associated with surface modification of QDs also retard their applications in live-cell systems. Therefore, novel materials that overcome the stability and toxicity issues in live-cell imaging are in high demand.[7–10]

Conjugated polymers (CPs) are attractive materials that meet the optical requirements suitable for fluorescence microscopic imaging.[11] CPs exhibit high fluorescence quantum yield, large extinction coefficients, and efficient optical signal transduction. In addition, the synthetic versatility of CPs allows a wide selection of functional groups and coupling chemistries for attachment of biological molecules. In spite of these promising properties, intrinsic hydrophobicity originating from the π-conjugated aromatic backbone limits the potential applications of CPs in biological systems. By introducing charged functional groups in the CPs' side chains, the detection of nucleic acids,[12] proteins,[13,14] bacteria,[15] or cancer cells[16] in vitro has been demonstrated.

Herein, we introduce fluorescent conjugated polymer nanoparticles (CPNs) that are capable of fluorescence imaging of live cells. CPNs are stable, nanometer-sized fluorescent particles fabricated by a simple solvent exchange in a CP solution. An amine containing poly(*p*-phenylene ethynylene) (PPE) was designed and fabricated into CPN in water to demonstrate live-cell imaging. The CPNs are cell permeable and accumulate exclusively in the cytosol without any measurable inhibition of cell viability. In addition, CPNs exhibit high resistance to photobleaching, in contrast to commercially available dyes.

A PPE was synthesized as a representative CP by the palladium/copper-catalyzed cross-coupling reaction in a mixed solvent of DMSO and morpholine (1:1 v/v). We designed monomers to minimize π-stacking of aromatic backbones in aqueous media by introducing amine groups at the end of ethylene oxide linkers (Scheme 1). Primary amine groups in the PPE are of particular interest because they increase aqueous solubility of PPE upon protonation and provide a site for coupling of biologically active molecules. Moreover, protonation of the amine group increases fluorescence intensity because of reduced chain–chain interactions.[17,18]



Scheme 1. Synthesis of PPE from diiodoarene **1** and diyne **2**. See Experimental Section for details.

We reported that the phase-inversion precipitation of PPE in a poor solvent allowed formation of stable particles.[19] PPE formed variously sized particles in aqueous phases, depending on both PPE concentration and salinity. Herein, sequential ultrafiltration with acetic acid, ethylenediaminetetraacetic acid (EDTA), and water were used for the fabrication of stable nanometer-sized particles. Acetic acid aided removal of metal-ion contamination (palladium and copper) and reduced PPE aggregation in the CPNs by generating repulsive forces. Features in the fluorescence spectrum, such as broadened emission, indicated that the particle formation was driven by aggregation of PPEs. However, CPN exhibits considerable quantum yields (QY; 0.17) in water, unlike other secondary- or tertiary-amine-containing PPEs, which exhibit very low QY in water owing to severe aggregation.[20,21] Dynamic light-scattering experiments indicated the formation of nanoparticles with an average size of 97 nm and polydispersity index of 0.13.[22] Transmission electron microscopic (TEM) images also indicate formation of nanoparticle clusters with broad

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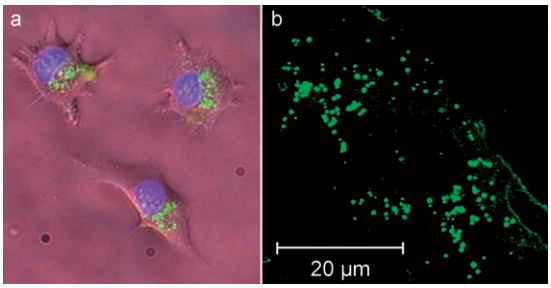

Figure 1. Fluorescence images of live (a) and fixed (b) cells. a) BALB/C 3T3 cells were incubated sequentially with CPNs (green) and Hoechst dye (blue). The image is a composite of two micrographs using GFP (for CPNs) and DAPI/Hoechst/AMCA (for Hoechst) filter sets. b) Live BALB/C 3T3 cells were incubated with CPNs and fixed for confocal microscopic study. Variously sized spherical dots were found in cytosol. In both cases, CPNs accumulated in the cytosol, not in the nucleus.

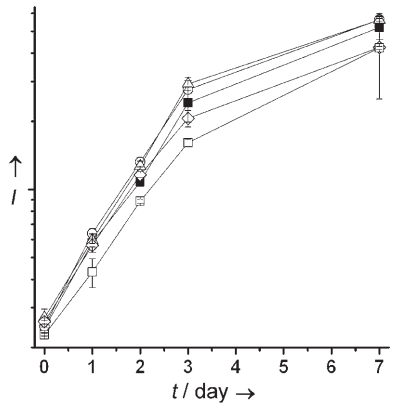

Figure 2. Cell viability results at various concentrations of CPN. BHK cells were incubated with various concentrations of CPN for up to seven days. ■ no CPN; ○ 33 μM of CPN; △ 66 μM; ◇ 132 μM; □ 264 μM of CPN.

size distribution resulting from various aggregations of CPNs in a TEM grid (see Supporting Information).

The CPNs were further diluted with a cell-culture media (Dulbecco's modified eagle media, Invitrogen) for live-cell imaging. Various cells, including baby-hamster kidney (BHK) and BALB/C 3T3 (mouse embryonic fibroblast), were incubated with the CPNs in culture media for various time periods (from one hour to several days) to examine the cell permeability, photostability, and cellular toxicity of CPNs. Figure 1 shows microscopic images of live BALB/C (a) and fixed 3T3 cells (b) stained by the CPNs overnight.[23] CPNs were found exclusively in the cytoplasm, and especially around the perinuclear region. To identify the location of the CPNs, we co-stained the cells with CPNs then a lysosome-specific fluorophore (LysoTracker Red DND-99, Invitrogen). The fluorophores with weakly basic amines are likely to accumulate in cellular compartments with low internal pH values, such as lysosomes.[24] Although CPN has many amine groups, it accumulated randomly throughout the cytosol, which was determined by the lack of overlap with the LysoTracker (see Supporting Information). It is noteworthy that fast photobleaching of the molecular fluorophore was observed during fluorescence imaging of the live cells, whereas CPNs had a fairly stable signal during the imaging (see Supporting Information for a stability comparison). A confocal microscopic study of fixed 3T3 cells further suggests that CPNs were accumulated in vesicular structures, such as early or late endosomes (Figure 1b).[25]

The quantitative CPN effect on cell viability was measured using a commercial viability assay kit. BHK cells were inoculated into 96-well cell-culture plates (Corning) at 500 cells per 100 μL, followed by incubation over time in the presence of varying amounts of CPNs. Live cells were quantified at various time points using the CellTiter-Glo assay kit (Promega Corp.), which measures ATP as an indicator of metabolically active cells. The results for the CPN-containing culture wells were compared to control wells incubated in the absence of CPNs. Figure 2 shows the average luminescence values for each time point with only minimal viability inhibition observed even at 264 μM of CPNs over the course of a one-week cell culture.

We compared the photostability of CPNs with representative fluorescent dye fluorescein. Live 3T3 cells were incubated with fluorescein-labeled TAT (FITC-TAT) followed by paraformaldehyde fixation. TAT is a peptide fragment (47–57) of HIV transactivator protein that penetrates cellular membranes.[26] Under a fixed confocal laser setup, fluorescence images of CPN and FITC were collected for 200 seconds at 6-second intervals by excitation at 488 nm. Normalized fluorescence intensity of individual images were plotted as a function of exposure time (Figure 3). CPN maintained fluorescence intensity over the exposure periods, whereas FITC exhibits fast photobleaching. Moreover, fluorescence intensity of CPN collected at 20-times-higher laser intensity than the normal operational laser power (4%) also exhibited no noticeable increase in the photobleaching (open circles in Figure 3); indicating CPN is a photostable fluorophore. It is not clear why CPNs possess better photostability than the FITC dye. We assume that when the nanoparticles

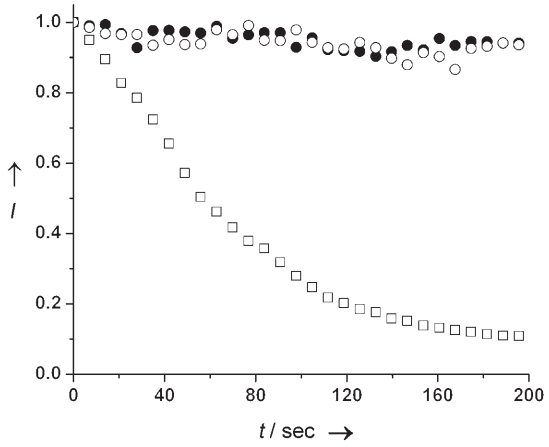

Figure 3. Photostability comparison of CPN (● and ○) compared to fluorescein (FITC, □). Fluorescence intensity is normalized to the initial intensity. ○ CPN excited at 80% of 488 nm laser power; ● CPN excited at 4% of the power; □ FITC excited at 4% of the power.

are formed, the hydrophobic side chains of PPE provide protective layers for the conjugated backbone against the diffusion of reactive oxygen, which causes photodegradation.[27]

In conclusion, we have presented a potential fluorescent nanoprobe with fluorescent conjugated polymers that is easily synthesized and has promise for studies in live cells with no viability inhibition. CPN has a reasonably high quantum yield and better photostability than representative fluorophores. Knowledge about both cellular uptake mechanism and location of CPNs will provide a foundation for various fluorescence-imaging-based applications including delivery of bioactive molecules in cells.

Experimental Section

Synthesis and purification of PPE: **1** (42 mg, 93.3 μmol) and **2** (50 mg, 93.3 μmol) were weighed into a 40-mL glass vial. CuI (12 mg) was weighed into a second vial, and both vials were transferred into the glove box. Morpholine was added to the CuI-containing vial to make a 10 mg mL^{-1} solution. [Pd(PPh$_3$)$_4$] (11 mg) was dissolved in morpholine to make a 5 mg mL^{-1} solution. DMSO (5 mL), morpholine (3.8 mL), some of the CuI solution (124 μL), and some of the [Pd(PPh$_3$)$_4$] solution (1.08 mL) were added to the vial containing the monomers. The vial was then placed in an 80 °C oil bath and vial contents allowed to stir for 16 h. The solution turned clear orange with some gels in solution and some on the vial wall at the meniscus. The probe was diluted with glacial acetic acid (300 mL), which dissolved all of the gel. Using a solvent-resistant stir cell fitted with a 10000-molecular-weight cut-off (MWCO) membrane, the solution was concentrated, combined with acetic acid (500 mL), and purified by filtration. It was then concentrated to approximately 50 mL, diluted into 300 mL of 0.1 mM EDTA, then dialyzed against of EDTA (500 mL, 0.1 mM). It was again concentrated to approximately 50 mL then diluted by adding to water (300 mL), and dialyzed against 1 L of water. The solution was finally concentrated to approximately 75 mL, filtered through glass wool (1.0 μm) and a syringe filter (0.45 μm). The filtrate was diluted to a final volume of 90 mL (0.6 mg mL^{-1} as determined by lyophilization). Yield: 80 %. ^{1}H NMR (400 MHz, D_2O): $\delta = 6.996$ (br s, 4H, aromatic), 4.266 (br s, 8H, PhOCH_2), 3.955–3.123 ppm (br m, 38H).

Cell viability measurements: BHK cell suspensions (5×10^3 cells mL^{-1}, or 0 cells mL^{-1} as a negative control) were prepared, seeded at 95 μL well^{-1}, in 96-well tissue culture (TC)-treated plates. Cells were incubated overnight (37 °C/5 % CO_2) to allow attachment to occur, and then spiked with CPN (5 μL, diluted in PBS) at varying concentrations to yield the desired final CPN concentration in culture (0, 2.5, 5, 10, 20 μg CPN per 100 μL well). Cells were then incubated (37 °C/5 % CO_2) and assayed at various times using the CellTiter-Glo assay kit (Promega). Four replicates at each CPN concentration were assayed at each time (1 hour, 1 day, 2 days, 3 days, and 4 days) according to the manufacturer's instructions (analysis by luminescence, Perkin–Elmer Victor 2 reader).

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