Simple conjugated polymer nanoparticles as biological labels

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The use of nanoparticles in biology, especially in cellular imaging, is extremely promising and offers numerous advantages over existing organic dye systems. There are, however, constraints that need to be addressed before the use of such materials in mainstream clinical applications can be realized. One of the main concerns is the use of metal-containing particles that are potentially toxic or interfere with other diagnostic processes. Here, we present the use of simple conjugated polymer nanoparticles as alternative photostable cellular optical imaging agents.

Keywords: nanoparticles; conjugated polymers; biological labelling

1. Introduction

Numerous reviews report the advantages of quantum dot biolabels, and two main criteria for the use of nanomaterials are emerging: prolonged photostability and non-toxic character (Alivisatos 2004; Derfus et al. 2004; Jaiswal & Simon 2004; Parak et al. 2005). Several studies have shown that cadmium-containing quantum dots exhibit cytotoxic behaviour attributed to the cadmium content (Kirchner et al. 2005; Ballou et al. 2007; Liang et al. 2007). Conjugated polymers are attractive alternatives—extremely luminescent materials made entirely of relatively benign constituents. The photo-physics of conjugated polymers are well understood owing to applications in light-emitting devices, solar cells and organic electronics (Sirringhaus et al. 1998; Brabec et al. 2001; Moliton & Hiorns 2004). However, most conjugated polymers are soluble only in organic solvents, a major limitation for their use in biological systems. Several groups have explored
the potential of conjugated polymers in biology, and induced hydrophilicity by introducing ionic or water-soluble side groups (Chen et al. 1999; Heeger & Heeger 1999; Kuroda & Swager 2003; Disney et al. 2004; Kumaraswamy et al. 2004). The need to design and synthesize specific water-soluble conjugated polymers eliminates the use of the wide range of readily available hydrophobic conjugated polymers, effectively removing a vast library of materials that emit across the visible and infra-red spectra. Here, we demonstrate cellular labelling using the simple, common conjugated polymer poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene] (MEH-PPV), an organically soluble material routinely used in light-emitting devices and structurally similar to the polymer used in the seminal report of a polymer light-emitting device (Burroughes et al. 1990). Rather than alter the structure of the polymer, we have prepared nanoparticles of MEH-PPV, using surfactants to passivate the surface and envelop the material while retaining the emitting character of the polymer.

2. Material and methods

The particles were prepared using a similar route to the one described by Landfester et al. (2002). Two materials were used as surfactants, sodium dodecylsulphate (SDS) and polyethylene glycol (PEG).

(a) Preparation of MEH-PPV nanoparticles

The particles were prepared using a reverse-micelle technique, modified from the reported Landfester method, replacing the surfactants with either SDS or PEG. In a typical reaction, 0.02 g of MEH-PPV (Sigma Aldrich, Gillingham, Dorset, UK, used as-received) was dissolved in 8.6 g dichloromethane. In a separate flask, 0.04 g PEG (molecular weight 8000, Sigma Aldrich, used as-received) was dissolved in 30 ml deionized water. The polymer solution was added slowly through a pipette to the rapidly stirring PEG solution, and finally covered. The solution was allowed to stir for 1 h, and then sonicated for 2 h. The solution was then allowed to stand for one week in the dark, followed by centrifugation at 7000 r.p.m. for 2 h. The resulting clear red solution was then filtered, stored in the dark and used as required. To prepare SDS-capped particles, 0.05 g of MEH-PPV was used and the PEG was substituted with 0.15 g of SDS (Sigma Aldrich, used as-received).

(b) Labelling CHO-K1 (Chinese hamster ovary) cells

CHO-K1 cells (ATCC, USA) were grown in cover slips in F12K medium (Gibco; Invitrogen, Paisley, UK), supplemented with 10 per cent fetal calf serum (FCS), penicillin (5 U ml\(^{-1}\)) and streptomycin (50 mg ml\(^{-1}\)) at 37\(^\circ\)C and 5 per cent CO\(_2\). On the day of particle uptake, the medium was replaced with Optimem basic medium (Gibco; Invitrogen) without serum or antibiotics. Particle cell-uptake experiments were performed in 24-well plates, and conjugated polymer MEH-PPV particles coated with SDS surfactants were prepared by mixing 2 \(\mu\)l of lipofectamine 2000 (Invitrogen) with \(10^{10}\) particles ml\(^{-1}\) carboxyfluorescein-labelled latex nanoparticles of 100 nm (fluospheres; Invitrogen) and/or an equivalent amount of conjugated polymer nanoparticles in Optimem, followed by sonication for 5 min.
The mixture was then added onto cells and left for 2 h at 37°C and 5 per cent CO₂. After 2 h, the cells were washed with PBS, and fixed with 4 per cent paraformaldehyde (PFA) at 37°C for 20 min. Cells were further washed with PBS and incubated with 0.5 per cent glycine for a further 20 min. DAPI was added (300 nM in PBS) to stain the nucleus blue. After a final wash with PBS, cells were mounted on microscopic slides and were subject to fluorescent microscopy (Nikon Eclipse E600). Cells were visualized for 1 h continuously under fluorescence (FITC filter for fluorospheres 488 nm) and red filter (excitation 510–560 nm) for capped particles, with photos taken at regular intervals using a Nikon digital camera (DXM1200). For DAPI staining, visualization using a filter with an excitation spectrum of 330–380 nm was used.

(c) Labelling h-TERT human fibroblast cells and clathrin measurements

Infinity telomerase immortalized primary human fibroblasts (h-TERT BJ1 human fibroblasts; Clonetech, Mountain View, CA, USA) were cultured on 13 mm glass coverslips at a density of 1 × 10⁴ cells per dish in 1 ml of complete medium for 24 h to allow attachment. The cells were then incubated with the nanoparticles (diluted in a cell medium) for 60 min. After incubation, the cells were fixed in 4 per cent formaldehyde/PBS, with 1 per cent sucrose at 37°C for 15 min. The samples were then washed with PBS, and permeabilizing buffer was added at 4°C for 5 min. The samples were then incubated at 37°C for 5 min in 1 per cent BSA/PBS. This was followed by the addition of anti-tubulin/anti-clathrin/anti-dynamin primary antibody (1:100 in 1% BSA/PBS, monoclonal anti-human raised in mouse (IgG1); Sigma, Biogenesis and Upstate Biotechnology, respectively) for 1 h at 37°C. Simultaneously, rhodamine-conjugated phalloidin was added for the duration of this incubation (1:100 in 1% BSA/PBS; Molecular Probes). The samples were then washed in 0.5 per cent Tween 20/PBS and a secondary, biotin-conjugated antibody (1:50 in 1% BSA/PBS, monoclonal horse anti-mouse (IgG); Vector Laboratories, UK) was added for 1 h at 37°C followed by more washing. Finally, FITC-conjugated streptavidin was added (1:50 in 1% BSA/PBS; Vector Laboratories) at 4°C for 30 min, followed by a final wash. Any excess particles were washed away during the staining process. Samples were viewed using an inverted Zeiss Axiovert (560 ± 20 nm notch filter) and images were captured via Visicapture (Scion, Frederick, USA) on an e-mac G4.

The cells were fixed with 1.5 per cent glutaraldehyde (Sigma) buffered in 0.1 M sodium cacodylate (Agar, UK) (40°C, 1 h) after incubation with the nanoparticles. The cells were then post-fixed in 1 per cent osmium tetroxide for 1 h (Agar) and 1 per cent tannic acid (Agar) was used as a mordant. Samples were dehydrated through a series of alcohol concentrations (20, 30, 40, 50, 60, 70%), stained in 0.5 per cent uranyl acetate, followed by further dehydration (90, 96, 100% alcohol). The final dehydration was in hexamethyl-disilazane (Sigma), followed by air-drying. Once dried, the samples were coated with gold before examination with a Hitachi S800 field emission SEM at an accelerating voltage of 10 keV.

3. Results and discussion

The particles of MEH-PPV were found to be approximately 100 nm in diameter, (electronic supplementary material, figure S1). Many applications use particles in this size regime, notably for blood flow analysis, neuronal tracing as described by
The absorption and emission profiles of the polymer particles differed slightly from the parent polymer in dichloromethane (figure 1), because of the change in the spatial environment for the polymer upon transformation into a hydrophilic particle, similar to previous reports on the formation of polymer aggregates in different solutions (Nguyen et al. 1999; Traiphol et al. 2007) and in the formation of thin films (Nguyen et al. 2000). Broadening of the absorption spectrum is seen, with a red band edge of ca 600 nm (as opposed to ca 560 nm) and a slightly shifted blue onset. This was caused by a spread in the individual chromophore conjugation lengths from coiling or twisting of the constituent polymer chains brought on by their transition from a solution to a particulate environment. With these different chromophores absorbing, a wider absorption profile is obtained, enveloping all those absorption bands (Traiphol et al. 2006).

The emission maxima red shifted from 559 to 594 nm upon particle formation while maintaining the overall shape of the emission profile (figure 2a(i,ii), b(i,ii), c(i,ii)). The spectral red shift indicates the aggregation of polymer chains, which would be expected when the chains are processed out of a solution into particles.
With reference to previous reports by Schwartz (2003), we suggest the red shift in emission is due to increased overlap of $\pi$-orbitals as the polymers are aggregating in the particles, resulting in delocalization of the $\pi$-electrons across several chains. This delocalization enabled the formation of novel electronic species with lower band gaps. Although there might be coiling effects shortening effective conjugation length in the individual chains this is probably overcome by this orbital overlap during aggregation (Traiphol et al. 2006).

The emission is relatively narrow owing to the migration of energy states along the backbone to the lowest energy segments, which then emit (Padmanaban & Ramakrishnan 2000). The lower energy shoulder in the emission spectra corresponds to the relaxation of the $\pi$-electrons through a ground-state vibrational energy level, and is more pronounced in the particle emission spectrum.

There was also a reduction in emission quantum yield (measured at 4.4% against rhodamine 6G) owing to the formation of non-radiative species (exciton–exciton annihilation) across the polymer chains because of tight packing of the polymer in the particle; again, previously observed in aggregates of MEH-PPV (Nguyen et al. 2000). While a high quantum yield is clearly desirable for biological labelling, the reduced emission is still sufficient for biomolecular labelling purposes.

To investigate the potential for labelling, SDS-capped MEH-PPV particles, approximately 100 nm in diameter, were incubated with CHO-K1 (Chinese Hamster ovary) cells. The particles were compared for their endocytosis with similarly sized (100 nm) fluorescently labelled nanoparticle fluospheres (carboxyfluorescein latex nanoparticles). Neither SDS-capped MEH-PPV particles nor fluospheres were able to permeate the cell membrane avidly and label the cells over 2 h. The particles were therefore assisted in their cell entry by a small amount of transfection reagent, which assisted cell membrane permeation. Both sets of particles labelled the cells effectively (figure 2a–c), although the carboxyfluorescein-labelled particles appeared to label cells faster than the SDS-capped polymer spheres. As the particles were approximately the same size and their endocytosis assisted by cationic lipids, it might be expected that they would show similar levels of cell uptake over time, although the cellular uptake of nanoparticles is known to be clearly affected by the surfactant surface chemistry.

The photostability of an imaging agent is a major issue, with the majority of organic dyes fading over a relatively short period of time due to photobleaching. A comparison of the emission from cells labelled with the MEH-PPV particles (SDS surfactant) and 100 nm Invitrogen polystyrene spheres doped with carboxyfluorescein under constant illumination showed a gradual bleaching in the cells containing fluospheres, while cells labelled with the MEH-PPV particles were, surprisingly, not only stable but, in fact, increased in brightness over a 62 min period (electronic supplementary material, figure S2). The increasing brightness is attributed to UV photo-curing or J-aggregate formation in the conjugated polymer systems, as theorized by Siddiqui & Spano (1999). MEH-PPV particles appeared to accumulate around the nucleus whereas fluospheres remained dispersed in the cytosol. Fluospheres appeared more aggregated than MEH-PPV particles inside the cells where distinct fluorescence could be

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seen in the case of MEH-PPV particles (electronic supplementary material, figure S3).

The SDS-capped MEH-PPV particles did not appear to affect cell membrane integrity for the 2 h period of co-incubation with the cells. However, longer exposure times (i.e. 4 h) resulted in cell membrane disruptions. Further investigations showed that the surfactant SDS is toxic to the cells, and that the observed cytotoxicity can be attributed to excess SDS in solution in combination with lipofectamine.

Further experiments explored the use of particles capped with PEG, known to be an effective and inert capping agent, in labelling h-TERT human fibroblast cells. After incubation for 1 h, the particles were clearly observed throughout the cluster of cells (electronic supplementary material, figure S4), which appeared healthy, with clear nuclei. The particles appeared to be mainly inside cells and around the nuclear area in agreement with the study in CHO-K1 cells. In control sets of cells (i.e. no particles present) no such dots were visible. To explore the mechanism of particle uptake and their suitability in cells, investigation into clathrin levels were undertaken (figure 3a,b). In figure 3a, a strong cellular uptake can be seen as evidenced by the strong green clathrin emission, suggesting uptake via clathrin-mediated endocytosis. F-actin staining (red) demonstrated a normal cell morphology, with clear stress fibres present, indicating that the cells are generally unaffected by the presence of the particles. Figure 3b,c indicates high levels of clathrin at the cell periphery and perinuclear region, highlighting the path of uptake, i.e. that the cell endocytosed the particles from the periphery, as evidenced by the green staining in the cell filopodia. The material was then moved internally via microtubules towards the nucleus for cell sorting, again
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Figure 3. (a) A h-TERT human fibroblast cell after exposure to MEH-PPV particles, the strong clathrin presence (indicated by the green colour) indicates a healthy cell undergoing strong endocytosis; (b) and (c) images which show the path of uptake, i.e. that the cell uptakes the particles from the periphery (the green staining in the cell filopodia), and the material is then internally moved via microtubules towards the nucleus for cell sorting; (d) SEM image of a single PEG-coated MEH-PPV nanoparticle on a healthy cell.

highlighted as the green area surrounding the nucleus. These images confirm the particles are motile and the healthy state of the cells suggests that the particles were not cytotoxic, although further studies are being undertaken. In figure 3d, a typical particle was imaged on a cell surface. The cell membrane appeared as control cells, with no obvious aberrations and interaction with the particles.

4. Conclusion

In conclusion, we have prepared nanoparticles of a simple conjugated polymer, and used them in simple labelling experiments. This simple method potentially allows a wide range of solid-state materials to be used in bio-imaging experiments, providing a new material technology that circumvents some highlighted issues regarding nanomaterials in biology. Further investigations, notably standard toxicity studies, are being undertaken and will be reported elsewhere.

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