Synthesis, characterisation and intracellular imaging of PEG capped BEHP-PPV nanospheres†

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Aqueous dispersions of poly(ethylene glycol) (PEG) capped poly[2-(2′,5′-bis(2″-ethylhexyloxy)phenyl)-1,4-phenylene vinylene] (BEHP-PPV) nanospheres with an average particle diameter of 13 nm have been synthesised by a miniemulsion route and used in simple intracellular imaging experiments.

Fluorescence microscopy is an important tool for the study of the various molecules involved in cellular function.[1] Traditional probes such as organic fluorophores and fluorescent dyes tend to suffer from rapid photobleaching;[2] however, advances in the synthesis of fluorescent nanoparticles have yielded some exciting candidates to circumvent this problem. A prominent example is quantum dots (QDs) which have long been considered promising for *in vitro* and *in vivo* fluorescence imaging.[3,4] However, questions remain over their suitability as biolabelling agents, as they contain toxic materials. Many studies have been performed on the cytotoxicity of QDs and whilst some groups have found that under certain conditions no short term toxicity is observed in live animals[5,6] and in cells,[7] other have found that CdSe cores are acutely toxic to cells, either by release of cadmium ions,[8] free radicals or surface interaction.[9–11] Even if the biocompatibility is increased with the addition of relatively benign inorganic shells or ligands it will be difficult to make them under the renal clearance limit of 5.5 nm and long term toxicity will be a real concern.[12] Given these limitations it is important that new probes are considered for *in vitro* and *in vivo* fluorescence imaging.

Conjugated polymers are organic semiconductors. Their conjugated backbone gives rise to π-electron delocalisation and an electronic band structure; the π–π* electronic transition allows the formation of excitons which facilitate photo- and electroluminescence.[13] They possess the ease of processing of plastics and the electronic behaviour of metals and semiconductors, and are well understood due to their application in light-emitting devices.[14] Conjugated polymers have high quantum yields and extinction coefficients in solution, and therefore exhibit high fluorescence brightness.[15] Importantly, they are relatively benign and should overcome some cytotoxicity problems associated with QDs. For these reasons, they are considered to be potentially useful in the production of fluorescent nanoparticles for use in fluorescence imaging.[16] The aggregated phase of PPE-type polymers has previously been explored, and may be seen as one of the seminal systems leading to our current work.[17] Nanoparticles containing hydroxyphenyl-benzoxazole derivatives have also been produced, with potential application in immunofluorescence labelling.[18]

Although most conjugated polymers are hydrophobic, it is possible to synthesise semiconductor polymer nanospheres (SPNs) with a hydrophilic surfactant to allow dissolution in water. Here, we have demonstrated imaging of HEp-2 cells using poly(ethylene glycol) (PEG) capped poly[2-(2′,5′-bis-(2″-ethylhexyloxy)phenyl)-1,4-phenylene vinylene] (BEHP-PPV) SPNs. The SPNs were synthesised by a route similar to that of Landfester *et al.*[19] The hydrophobic polymer was converted into hydrophilic nanoparticles by a micelle-type route with PEG as a surfactant. BEHP-PPV has high thermal stability[20] and large side chains containing terphenyl units, which allow dissolution in common organic solvents and restrict contact between adjacent polymer backbones, increasing quantum efficiency.[21] The PEG passivated the surface and enveloped the particles, and the emitting character of the polymer was maintained. Although PEG contains no functional groups it should be possible to use functionalised surfactants to facilitate conjugation with enzymes, antibodies, proteins, *etc*. The incorporation of PEG onto the SPN surface has been shown to reduce clearance from the bloodstream by the reticulo-endothelial system (RES).[22] Furthermore, it has recently been demonstrated that positively charged PEGylated nanoparticles are internalised rapidly by HeLa cells using a clathrin-mediated endocytic pathway.[23]

The SPNs were synthesised by a miniemulsion route.‡ BEHP-PPV was dissolved in dichloromethane, mixed with an aqueous solution of the surfactant PEG and then sheared in an ultrasonic bath. The particle size was controlled by varying the polymer concentration in solution. Using a low concentration BEHP-PPV solution (20 ppm) an average particle size of 13 nm was obtained with a standard deviation of 5.4 nm as measured by electron microscopy (Fig. 1). The absorption and emission profiles of the SPNs differed slightly from those of the parent polymer in dichloromethane (Fig. 2). The absorption of the SPNs in water is wider with a flattened peak at 400 nm. This suggests there was a widening of the conjugation length distribution brought on by confinement of the polymer chains in the particles. Despite this change in the absorption characteristics there is no significant change in the emission profile. The red shift from the DCM solution to the aqueous dispersion is only 3 nm. Although all of the chromophores (chain segments) absorb photons, a process of energy funnelling occurs where absorbed energy is transferred

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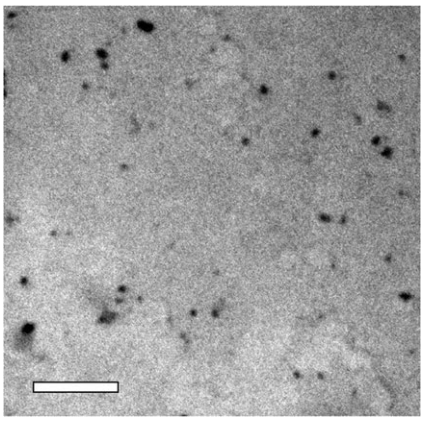

Fig. 1 Electron microscope image of BEHP-PPV polymer nanoparticles. Scale bar represents 200 nm.

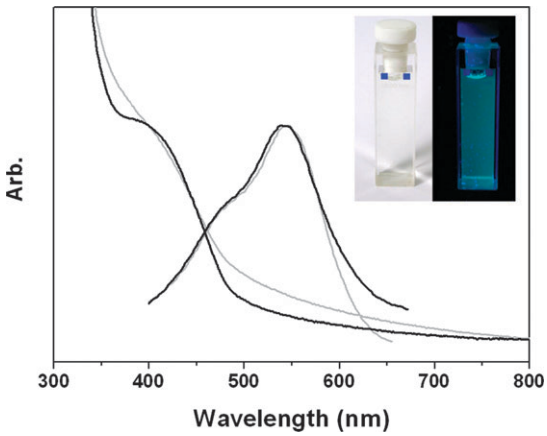

Fig. 2 Normalised absorption and emission spectra of BEHP-PPV in dichloromethane (black lines), and PEG-capped BEHP-PPV particles (grey lines). The inset shows the clear sample solution, and the sample under 365 nm UV excitation.

down to the lowest energy segments by FRET prior to emission. This is consistent with previous reports on the behaviour in films of the structurally similar polymer MEH-PPV.[24]

The quantum yield of the BEHP-PPV particles in water was measured as 2.7% by comparison with a fluorescence standard, Atto 390. Although this yield is low, when we consider the relatively large extinction coefficient ($>3 \times 10^6$ M^{-1} cm^{-1}), we see that the high fluorescence brightness required for imaging is maintained. Fig. 2 (inset) shows an image of the SPNs which were synthesised from a 20 ppm polymer solution. Under UV excitation the optically clear sample is clearly fluorescent. The reduction in quantum yield from solution to SPN is likely to be because of an increased number of structural defects in the polymer due to conformational changes brought on by chain confinement. Excitons can move to defects where they decay non-radiatively to the ground state.[25] Additionally, increased overlap of π-orbitals can result in the formation of electronic species, such as excimers and aggregates, which quench emission.[13] In our imaging study the internalisation of the BEHP-PPV particles into cultured HEp-2 cells was measured after 24 hours (Fig. 3). The uptake of the particles was seen after 1 hour (data not shown), although the internalisation

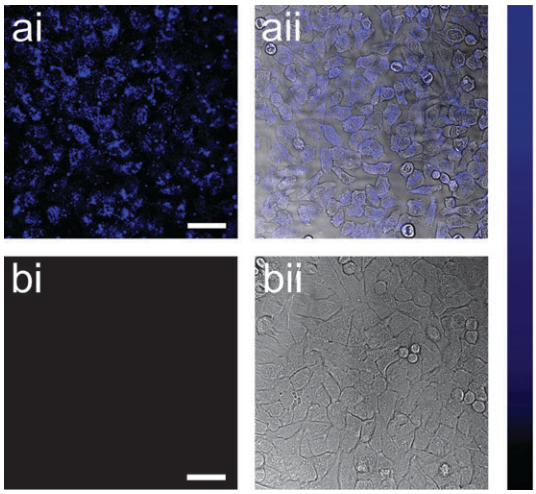

Fig. 3 The uptake of BEHP-PPV particles into cultured cells. HEp-2 cells were incubated with BEHP-PPV particles for 24 hours (panel a) and were found to be concentrated in the cytoplasm. Panel (b) shows cells which were not incubated with the particles as a control and also left for 24 hours. (ai) and (bi) show the fluorescence response; (aii) and (bii) show the fluorescence overlay with the bright field images. Scale = 50 μm.

appeared to occur exponentially over time. The particles appeared to be more concentrated throughout the cytoplasm although some uptake into the nuclei was evident, indicating that they were small enough to pass through the nuclear pore complexes by passive diffusion. It has previously been demonstrated using colloidal gold nanoparticles that the cutoff for passive diffusion into the nucleus is approximately 9 nm[26] and so the partial uptake of the BEHP-PPV particles seen here is not unexpected. This has implications for the future use of these nanoparticles in a field such as cancer research where nanoparticles are currently being investigated as a drug carrier capable of directly localising and releasing drugs into cell nuclei. It has been previously shown that the conjugation of 40 nm superparamagnetic iron particles to the TAT peptide sequence from HIV-1 allows extremely efficient uptake of these particles and this system could be employed here.[27]

As a measure of any potential cellular toxicity induced by BEHP-PPV SPN uptake and to demonstrate that particle uptake was not due to damaged cell membranes, HEp-2 cells were incubated with calcein red–orange, AM. This is well retained by live cells that possess an intact plasma membrane whereas their products rapidly leak from dead or damaged cells. Here it was shown that cells incubated with BEHP-PPV SPNs retained the fluorescent products equally as well as the cells incubated without the particles over a 2 hour time period (ESI†, Fig. 1).

This indicated that the plasma membrane remained intact and had not been compromised by particle uptake. Based on this evidence we suggest that BEHP-PPV SPNs are promising alternatives to fluorophores with toxic constituents. It was also found that the particles did not show any significant photobleaching over a time period of ~5 minutes when using a 405 nm, 25 mW diode laser at 50% and acquiring images every 1.635 seconds (ESI†, Fig. 2), demonstrating again the potential use of BEHP-PPV particles as biological markers.

Furthermore, HEp-2 cells which had been fixed in 70% MeOH and 30% acetone and stored for 1 month showed exactly the same fluorescence intensity as the initial study, indicating that cells labelled with these particles can be stored indefinitely without any loss in sensitivity.

In conclusion, we have shown that BEHP-PPV SPNs are stable and bright biological markers which do not compromise cell viability. Whilst these results are simplistic in nature, a more useful application such as flow cytometry is envisaged. They offer many of the advantages of quantum dots whilst eliminating the need for toxic constituents. The synthesis was simple and easily reproducible, and the SPNs were shown to have favourable photoluminescence characteristics. They have potential for use in live cell imaging and dynamics, and *in vivo* diagnosis and treatment of specific diseases, such as cardiovascular disease and cancer. To enhance targeted delivery, the surface functionality of the particles could be altered to deliver the material to specific sites using antibodies or specific proteins conjugated to the surface.

Notes and references

‡ *Preparation of BEHP-PPV nanoparticles*: 0.42 mg BEHP-PPV (minimum molecular weight 30 000) were added to 16 ml dichloromethane to make a 20 ppm solution, and stirred until completely dissolved. 74 mg of poly(ethylene glycol) (PEG 8000) were dissolved in 20 ml deionised water and stirred for 10 minutes. The two immiscible solutions were combined in a beaker. This was put inside a 35 kHz ultrasonic bath. The initial temperature was ambient, around 20 °C. Heating and sonication were started simultaneously. With the temperature approaching 55 °C at 25 minutes, the whole reaction mixture turned clear and there was no further change. The transparent aqueous solution was filtered through filter paper to remove solid residue, including a thin crust of polymer. The solution was centrifuged, and then refiltered. A transparent yellow solution was obtained which glowed green under UV excitation. The quantum yield was measured relative to a fluorescence standard Atto 390 (Fluka 89313). The solution was stable for months. All the chemicals were obtained from Sigma-Aldrich, Poole, UK.

BEHP-PPV uptake into HEp-2 cells and photobleaching studies: HEp-2 cells, cultured in Eagle minimum essential medium (MEM) supplemented with 10% heat inactivated foetal bovine serum, 0.1 mM non-essential amino acids and 2 mM L-glutamine (all from Sigma-Aldrich, Poole, UK), were grown on sterilised 25 mm borosilicate round glass coverslips (thickness 1, VWR International, Lutterworth, UK) in sterile polystyrene 35 × 10 mm culture dishes (Corning, NY, USA). Cells were incubated with BEHP-PPV particles for 24 hours and then washed in 1× HBSS (with calcium and magnesium, without phenol red (Sigma-Aldrich, Poole, UK)). Finally the cells were fixed in 70% MeOH and 30% acetone for 10 minutes, washed in PBS (pH 7.2) and mounted onto glass slides with fluorescent anti-fade mounting medium (Dako, Ely, UK).

To investigate the effect of BEHP-PPV particles on cell viability, particles were loaded into HEp-2 cells for 24 hours and washed in 1× HBSS. One culture dish was not incubated with the particles to act as a control. Both sets of cells were then loaded with 4 μM CellTrace™ calcein red-orange, AM (Invitrogen, Paisley, UK) in MEM for 30 minutes and again washed in 1× HBSS. To determine cell viability, cells were imaged every 30 minutes for 2 hours to determine any loss of calcein red–orange fluorescence from damaged membranes.

All images were acquired on an inverted Leica DMIRE2 confocal microscope using a 40 × 0.75 lens and imaged using a TCS SP2 system with 405 nm diode and 543 nm HeNe laser lines. Emission for the BEHP-PPV particles was collected between 420 and 550 nm, whilst calcein red–orange, AM, was collected at 580–650 nm. The photobleaching of BEHP-PPV particles was investigated by exposing the particles in solution to excitation light from the 405 nm, 25 mW diode laser every 1.635 seconds for 280 seconds.

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