ChemComm

Cite this: *Chem. Commun.*, 2011, **47**, 5524–5526

www.rsc.org/chemcomm

COMMUNICATION

A potent fluorescent probe for the detection of cell apoptosis†

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Received 24th November 2010, Accepted 14th March 2011
DOI: 10.1039/c0cc05158f

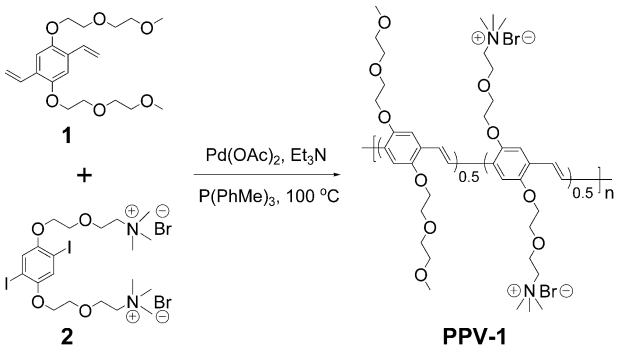
A new positively charged fluorescent probe poly(*p*-phenylene vinylene) derivative (PPV-1) has been developed for the detection of an important biological process, apoptosis, in a simple and fluorescent label-free way.

Apoptosis, programmed cell death, is an important biological process that naturally occurs during normal tissue development and homeostasis and plays a key role in a number of diseases (*e.g.* cancer and heart disease).[1] Several characteristic changes, including cytoplasmic blebbing, loss of cell membrane asymmetry and integrity, chromatin condensation, and chromosomal DNA fragmentation, will emerge when a cell is subjected to apoptosis.[2–4] Detection of apoptosis is of great importance in many areas of biological research; to some extent, apoptosis could be considered as an indicator for evaluating the efficacy of antitumor therapies and disease progression.[5,6] Up to date, numerous assays have been developed.[1,3] Among various methods, detection of phosphatidylserine (PS) exposing to the outer leaflet of the plasma membrane is one of the most commonly employed methods.[1,3,5–7] In normal cells, PS is strictly confined in the inner leaflet of the plasma membrane, which gives rise to its asymmetric distribution in cell membranes. However, loss of cell membrane asymmetry in apoptotic cells ultimately appears as the exposure of PS. Annexin V, a calcium-dependent protein, which specifically binds to negatively charged PS, is the currently used probe to visualize early-stage apoptosis. Despite the successful application of costly fluorescent-labeled Annexin V, there is still a call for exploiting novel, inexpensive and label-free detection reagents.

Fluorescent conjugated polymers, consisting of plenty of chromic repeat units, provide a novel platform for a variety of biological sensing applications. In particular, conjugated polymers can offer not only multivalent interactions towards biomacromolecules but also inherently sensitive fluorescent signals, leading to greatly enhanced detection sensitivity.[8–15] More recently, the possibility of conjugated polymers for cell imaging has been extensively probed.[16–20] In this contribution, we reported a new water-soluble poly(*p*-phenylene vinylene) derivative (PPV-1) which could be applied to the detection of cell apoptosis in a simple and fluorescent label-free manner. Moreover, the reported probe is compatible to commercial test equipments in biological research, such as confocal laser scanning microscopy (CLSM) and flow cytometry (FCM), exhibiting its potential value.

Synthetic route of PPV-1 is shown in Scheme 1. The reaction was based on Heck coupling between monomer 1 and monomer 2 with equivalent molar ratio,[21] which could provide the maximal percentage of positive charges to discriminate normal and apoptotic cells. Oligo(ethylene glycol) (OEG) side chains were designed to be an equally important component due to their ability to increase water solubility and their proven function of resisting nonspecific adsorption of biomolecules.[22,23]

The photophysical properties of PPV-1 were measured in water. PPV-1 exhibits an absorption maximum at 410 nm. The maximum emission wavelength is 514 nm with the fluorescence quantum yield of 1.9% (based on the polymer repeat units (RUs)). In spite of the relatively low quantum yield, the enrichment of the polymer at the target sites and the excellent light-harvesting capability altogether endow PPV-1 sufficient brightness to image apoptotic cells (*cf.* ESI†, Fig. S1). Furthermore, the broad absorption range (Fig. S2, ESI†) imparts the polymer a chance to share the same laser (488 nm) with the most commonly-used fluorescent dye—fluorescein isothiocyanate (FITC). Therefore, the new fluorescent conjugated polymer could be regarded as an analogue of FITC, which theoretically demonstrates its availability in the facilities suitable for FITC, especially CLSM and FCM.



Scheme 1 Synthetic route of PPV-1.

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† Electronic supplementary information (ESI) available: Details for synthesis of PPV-1 and experimental procedure, absorption and emission spectra of PPV-1, and control and comparative results of CLSM and FCM analysis. See DOI: 10.1039/c0cc05158f

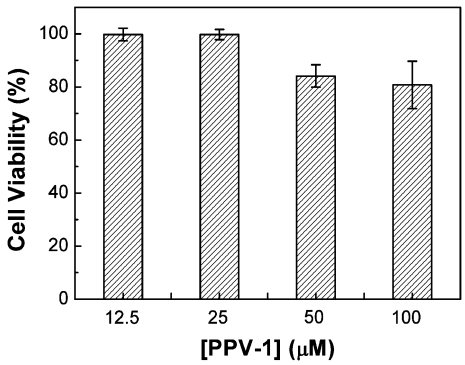

Fig. 1 Cell viability against various PPV-1 concentrations.

To investigate the biocompatibility of PPV-1, *in vitro* cytotoxicity test was conducted. We utilized the standard MTT assay to examine cell viability by incubating Jurkat cells with PPV-1 for 24 h. From the histogram displayed in Fig. 1, cell viability decreases slightly with the increase of PPV-1 concentration, particularly for more than 50 μM in RUs. However, the overall cytotoxicity of PPV-1 is not obvious; even when the concentration reaches 100 μM in RUs, cell viability still keeps at about 80%. But for practical applications, such a high concentration is impossible to be used; in other words, PPV-1 basically shows no obvious cytotoxicity and could serve as a cell-friendly imaging reagent.

Subsequently, our concern focused on the ability of PPV-1 to specifically recognize apoptotic cells. Even without external stimuli, apoptosis naturally occurs with an estimating percentage of 5%–10%.[5] In order to increase the proportion of apoptotic cells, anti-Fas monoclonal antibodies (mAb) were used for triggering apoptosis in a biological method. After harvesting by centrifugation, cells were incubated with PPV-1 in PBS at 37 °C for 15 min. The cells were then centrifuged and washed once with PBS to remove unbound PPV-1. Finally, the cell pellet was resuspended in PBS and preserved on ice for the following CLSM experiment and FCM analysis.

For fluorescence imaging, CLSM is an indispensable tool to gain precisely visual evidence on the specific binding of PPV-1 to apoptotic cells. As shown in Fig. 2a, the observed cell morphology keeps well and no characteristic changes were detected for the case of cells untreated with anti-Fas. However, the cells treated with anti-Fas (Fig. 2b) exhibit obvious morphological changes, *i.e.* cytoplasmic blebbing, especially clear in the local enlargement (Fig. 2c), which confirms the successful induction of apoptosis. It is easily observed that only the cells treated with anti-Fas emit bright green fluorescence (Fig. 2b and c); in contrast, due to the low apoptotic percentage, the cells without induction scarcely show detectable fluorescence (Fig. 2a). It should be noted that the background of collected fluorescent images is pretty clean for all the cases, indicating the potentiality of PPV-1 to be an ideal fluorescent probe. An important phenomenon could be found when one carefully examines the local enlargement shown in Fig. 2c: the more intact the cell morphology is, the less fluorescence is viewed and *vice versa*. All the above data reveal an indisputable fact that PPV-1 could selectively identify apoptotic cells. The mechanism for the selective response is proposed as follows. As mentioned previously, when a cell suffers from apoptosis, a

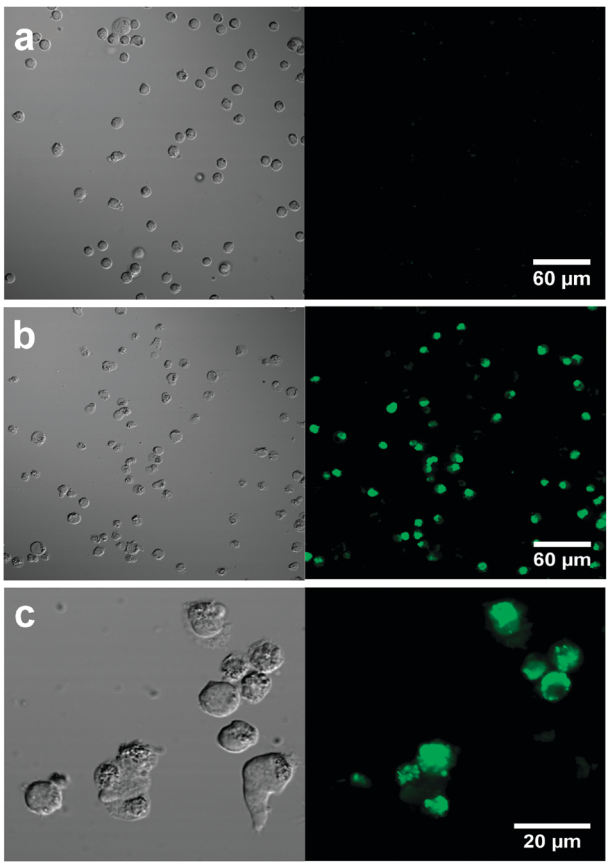

Fig. 2 (a) Fluorescence confocal microscopy images (CLSM) of Jurkat T cells induced without anti-Fas mAb but stained with PPV-1. (b) CLSM of Jurkat T cells induced with anti-Fas mAb followed by staining with PPV-1. (c) Local enlargement of the case in Fig. 2b. Left: bright field images, right: fluorescent images. The fluorescence of PPV-1 is highlighted in green.

series of changes will emerge, including the exposure of negatively charged PS, the enhancement of membrane permeability and so on. Consequently, positively-charged PPV-1 could easily bind to cell surfaces (owing to the increased negative charge density), and then the process of cell entry is facilitated. The fact that intact cells were seldom stained by PPV-1 is possibly attributed to the OEG side chains that could decrease the nonspecific interactions.

To further evaluate the robustness of PPV-1 as an apoptotic detection probe, the widely used instrument in biological research and clinical diagnosis, FCM, was included in our study to analyze cell apoptosis. Fig. 3 displays the obtained flow cytometry diagrams. In our experiments, cell fragments were excluded with forward and side-scatter gating to ensure that all detected signals originated from relatively intact cells. In each diagram, two separated regions could be seen, "negative" and "positive". The peak area of "positive" region represents the cell number associated with PPV-1 (*i.e.* apoptotic cells), while that of the "negative" region denotes the cell number without association with PPV-1 (*i.e.* relatively intact cells). The percentages designated in each diagram correspond to the proportion of "negative" and "positive" cells. All the cells in negative control were restricted to the "negative" region by adjusting photomultiplier tube (PMT) voltage in channel FL-1

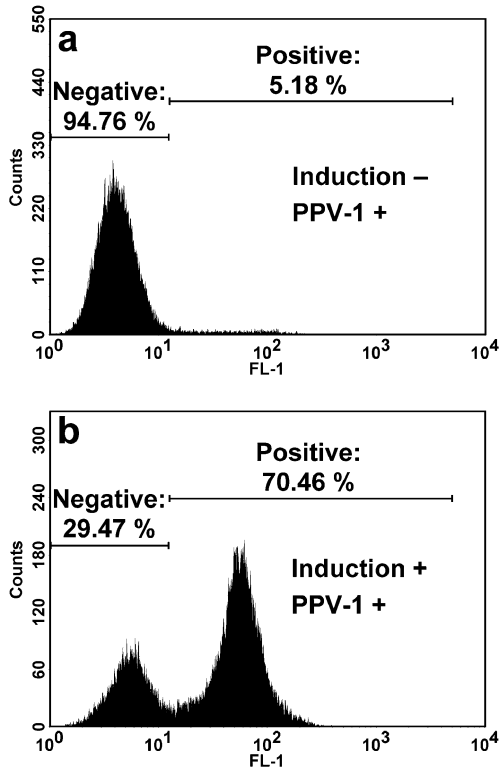

Fig. 3 Detection of apoptosis by flow cytometry. "Induction +" represents induction with anti-Fas, whereas "Induction −" represents no induction. "PPV-1 +" indicates incubation with PPV-1.

(Fig. S3, ESI†). Following that, the pre-prepared samples were analyzed using FCM. As shown in Fig. 3a, for the situation without treatment by anti-Fas, 94.76% cells belong to the "negative" region and the "positive" region only occupies 5.18%, consistent with the natural occurrence of apoptosis. The diagram in Fig. 3b, corresponding to the cells treated with anti-Fas, is markedly distinct from Fig. 3a. The "positive" percentage is as high as 70.46%, in well agreement with the results gained from CLSM; that is to say, both induction of apoptosis and discriminant staining are achieved successfully. On the basis of the data acquired from FCM, it can be concluded that PPV-1 is a potent fluorescent probe used for flow analysis, namely compatible with FCM.

To make a direct comparison between PPV-1 and Annexin V, we also conducted CLSM and FCM tests under identical conditions. The results shown in Fig. S4 and S5 (ESI†) demonstrate that PPV-1 basically exhibits the same capability as Annexin V towards the detection of apoptosis. It is emphasized that the induction time for Annexin V is relatively shorter than that of PPV-1, which indicates that PPV-1 is prone to detect mid- and late-stage apoptosis.[24] However, the main limitation of employing Annexin V lies in its requirement of fluorescent labeling which further increases the experimental cost.

In conclusion, a new positively charged fluorescent conjugated polymer (PPV-1) has been developed for the detection of cell apoptosis. The combination of the OEG side chains and half percentage of positively charges imparts PPV-1 the outstanding ability to discriminate apoptotic cells from normal cells. The properties of multivalent binding and photoluminescence render the polymer a specific and label-free probe to identify apoptotic cells, in avoidance of usage of expensive fluorescent labeled protein (Annexin V). Meanwhile, no obvious cytotoxicity is an additional advantage for its biological use. The most powerful capability of PPV-1 lies in its compatibility to commercial test equipments, demonstrating its further practicability in biological research. The reported work makes a contribution in the process of exploiting new fluorescent probes for the detection of apoptosis.

The authors are grateful to the National Natural Science Foundation of China (Nos. 20725308, 21033010, 90913014) and the Major Research Plan of China (No. 2011CB932302, 2011CB935800, 2011CB808400).

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