A Fluorogenic Probe for Cell Surface Phosphatidylserine Using an Intramolecular Indicator Displacement Sensing Mechanism

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Abstract: The detection of externalized phosphatidylserine (PS) on the cell surface is commonly used to distinguish between living, apoptotic, and necrotic cells. The tools of choice for many researchers to study apoptosis are annexin V-fluorophore conjugates. However, the use of this 35 kDa protein is associated with several drawbacks, including temperature sensitivity, Ca$^{2+}$ dependence, and slow binding kinetics. Herein, a fluorogenic probe for cell surface PS, P-IID, is described, which operates by an intramolecular indicator displacement (IID) mechanism. An intramolecularly bound coumarin indicator is released in the presence of cell surface PS, leading to a fluorescence “turn-on” response. P-IID demonstrates superior performance when compared to annexin V, for both fluorescence imaging and flow cytometry. This allows P-IID to be used in time-lapse imaging of apoptosis using confocal laser scanning microscopy and demonstrates the utility of the IID mechanism in live cells.

Phosphatidylserine (PS), an anionic phospholipid, is a minor, but important, component of the membrane of all eukaryotic cells. In healthy cells, PS is almost exclusively found on the inner (cytoplasm-facing) leaflet of the cell membrane. The exposure of PS on the cell surface is a common marker of cell death and one of the earliest hallmarks of apoptosis (programmed cell death), where externalization of PS acts as a signal for phagocytes to recognize and engulf the dying cells. The ability to selectively and rapidly detect apoptotic cells is crucial across a range of applications in molecular imaging and clinical medicine. To distinguish cell populations undergoing apoptosis from both living and necrotic cells, probes for the detection of cell surface PS are commonly employed in conjunction with nuclear stains such as propidium iodide. The most frequently used probe for detection of PS externalization is fluorescently labeled annexin V (AnV), a 35 kDa protein, which binds with high affinity to PS in a Ca$^{2+}$-dependent manner. However, there are a number of problems associated with the use of fluorescently labeled AnV derivatives to detect cell surface PS. These include binding of AnV to PS exhibits slow binding kinetics, which limit its use in high-throughput drug assays and must be performed at room temperature, precluding concomitant use of other assay systems, such as immunostaining. 2) the millimolar levels of Ca$^{2+}$ required for AnV binding to PS alter cellular physiology and can result in scramblases translocating more PS to the cell surface; and 3) currently available AnV-fluorophore conjugates are not so-called “turn-on” probes, and therefore their use generally requires a washing step to remove the unbound probe, so they are not useful for real-time imaging of apoptosis. To circumvent the numerous issues with using AnV-fluorophore conjugates for monitoring apoptosis, a fluorogenic small molecule probe capitalizing on intramolecular indicator displacement sensing has been developed.

Smith and co-workers have pioneered the use of zinc(II) dipicolylamine (ZnDPA) complexes to selectively target PS-rich membrane surfaces, typically using a bis(ZnDPA) PS binding motif attached to a fluorophore via a linker. However, the positioning of a linker between the fluorophore and PS binding site generally means that the fluorescence intensity is not altered upon binding, requiring a washing step to clear unbound probe prior to imaging. It is preferable for a fluorescent probe to only exhibit fluorescence upon binding to the analyte of interest, but fluorogenic probes for imaging apoptosis are scarce.

Conjugating a fluorophore to the binding site (receptor) of a probe to prepare a fluorogenic probe is often a synthetic challenge. One novel approach is to employ the highly effective, but rarely utilized, intramolecular indicator displacement (IID) mechanism in which a fluorescent indicator is covalently attached to the receptor via a flexible linker. In the resting state the indicator binds to the receptor site with quenching of its fluorescence. Displacement of the indicator from the receptor site by the analyte of interest leads to fluorescence (Figure 1).

Herein, a novel ZnDPA-based probe, P-IID, for the detection of apoptosis by sensing cell surface PS using a “turn-on” fluorescence IID activation mechanism is described. P-IID incorporates three components on a peptide backbone: a bis(ZnDPA) binding motif for PS; a 6,7-dihydroxyxocoumarin indicator positioned such that in the resting state the...
dye coordinates to one of the ZnDPA moieties, which quenches its fluorescence, and a stearic acid membrane anchor to reduce cellular uptake of the probe, thereby preventing the imaging of PS present on the inner leaflet of the cell membrane. In the presence of externalized PS, the fluorophore is displaced from the ZnDPA site by the anionic PS headgroup, which leads to restoration of the coumarin fluorescence (Figure 1).

The synthesis of P-IID was readily achieved using standard solid-phase peptide synthesis methods, with on-resin attachment of both the coumarin fluorophore and DPA moieties (see the Supporting Information for details). Complexation of the purified peptide with two equivalents of Zn$^{2+}$ led to fluorescence quenching (Supporting Information, Figure S1). This was attributed to the coordination of the catechol to one of the ZnDPA arms with the associated deprotonation of the catechol moiety, as indicated by DFT calculations (Supporting Information, Figure S2) and confirmed by mass spectrometry ($m/z$ 723.7886; C$_{71}$H$_{93}$N$_{15}$O$_{10}$Zn$_{2}^{2+}$).

The ability of P-IID to detect PS-rich membranes was demonstrated using vesicle titration experiments. Zwitterionic vesicles composed of 100% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), and anionic vesicles composed of 50% POPC and 50% 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) were prepared by standard extrusion techniques. Subsequent titrations of P-IID (10 μM, HEPES buffer pH 7.4) with the zwitterionic POPC vesicles resulted in negligible modulations in the fluorescence spectrum (Figure 2A), indicating no significant binding to the membrane surface, whereas the addition of anionic POPS vesicles caused an approximately four-fold fluorescence increase at around 530 nm (Figure 2B), attributed to the displacement of the coumarin indicator from the ZnDPA binding site by PS.

Having demonstrated that P-IID recognizes PS with a fluorogenic response, we set out to visualize apoptosis in both fixed and live cell fluorescence imaging studies. Initial experiments were conducted using HeLa cells in which apoptosis was induced by treatment with camptothecin (CPT, 40 μM, 12 h). Following CPT treatment, cells were stained with P-IID (20 μM, 20 min) and a commercial cell stain (SYTO85 Orange, 25 nm, 20 min). Confocal microscopy images show staining of apoptotic cells by P-IID, while healthy control cells remained unstained (Figure 3). Subsequent experiments in HCC-1806 cells showed that the observed membrane staining pattern was maintained across different cell lines (Supporting Information, Figure S3). Two

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**Figure 1.** Chemical structure of the probe P-IID and proposed binding mechanism with PS. The NO$_3^-$ counterions have been omitted for clarity.

**Figure 2.** A), B) Fluorescence spectra of P-IID (10 μM, HEPES buffer, pH 7.4, $\lambda_{ex} = 405$ nm) upon the incremental addition of (A) zwitterionic vesicles (100% POPC) or (B) anionic vesicles (50% POPC/50% POPS), and change in fluorescence intensity (F.I.) at 530 nm as a function of phospholipid concentration.

**Figure 3.** Confocal microscopy images of untreated and CPT-treated HeLa cells (focused on single cell). Cells were stained simultaneously with SYTO85 Orange (cell stain) and P-IID. Scale bars: 10 μm.
analogues of P-IID that lack the membrane anchor, bearing either a coumarin that does not coordinate to the ZnDPA binding site (and is therefore always “on”; probe 6, structure in Supporting Information), or the same fluorophore as P-IID (probe 8, structure in Supporting Information) were used as controls; neither of these showed the membrane staining observed with P-IID (Supporting Information, Figure S3).

Having shown the suitability of P-IID for fluorescence imaging of PS expression in apoptosis, we next assessed its performance in detecting apoptotic PS expression in HCC-1806 and MCF-7 cells, as compared to annexin V, using flow cytometry. Following induction of apoptosis by CPT treatment, cells were stained with P-IID (15 μM). Co-staining with propidium iodide (PI, 1 μg/mL) was performed to identify healthy cells (PI−/P-IID−, Q3), early apoptotic cells (PI+/P-IID−, Q4), late apoptotic cells (PI+/P-IID+, Q1), and dead cells/debris (PI+/P-IID+, Q2) in the flow cytometry profiles (Figure 4A, left two graphs; see Supporting Information for staining conditions). This is comparable to the commonly employed method of double labeling with AnV and PI.[20] In parallel experiments, cells were stained with commercially obtained AnV–Alexa Fluor 647 (2.5 μg/mL−1) and PI. The flow cytometric profiles of apoptotic cells stained with P-IID resembled those stained with the AnV–Alexa Fluor 647 conjugate when the recommended standard conditions for AnV staining (15 min, 2.5 mM Ca2+, 25°C, washing step after staining) were used (Supporting Information, Figures S5 and S16, left two graphs), indicating that there is little difference in the frequency of cells binding to either AnV or P-IID, under these conditions.

To determine whether P-IID would be effective in conditions under which AnV staining is problematic, such as when the labeling of apoptotic cells is required in the absence of Ca2+, without washing steps, or at low temperatures (i.e. on ice), a range of experiments were conducted in which the standard conditions were modified accordingly, and the results were quantitatively compared to those obtained with AnV (Figure 4A–E for HCC-1806 cells, Supporting Information, Figures S5–S10 for MCF-7 cells). Figure 4A shows representative flow cytometric profiles of healthy, and apoptotic HCC-1806 cells stained with P-IID and PI, in the presence, and absence of 2.5 mM Ca2+. No significant difference in the frequency of cell populations was observed between CPT-treated cells stained with P-IID in either the presence or absence of 2.5 mM Ca2+ (Figure 4B), indicating that P-IID can effectively detect apoptotic cell populations in the absence of Ca2+ ions. Similar results were obtained when the experiment was performed with MCF-7 cells (Supporting Information, Figure S5). In contrast, AnV only stained apoptotic cell populations in the presence of Ca2+ ions (Figure 4C for HCC-1806 cells; Supporting Information, Figure S6 for MCF-7 cells).

We next evaluated if a washing step was required after staining either HCC-1806 or MCF-7 cells with P-IID or AnV. P-IID stained
apoptotic cells equally well, regardless of either the cell type and whether or not a washing step had been performed (Figure 4D, left graph for HCC-1806 cells and Supporting Information, Figure S7 for MCF-7 cells). This was ascribed to the “turn-on” nature of the probe. In contrast, while there was no significant difference in AnV staining of HCC-1806 cells when compared to the standard conditions (Figure 4D, right graph), with MCF-7 cells “no-wash” AnV staining showed higher numbers of early apoptotic as well as late apoptotic cells (Supporting Information, Figure S8), suggesting that non-specific staining had occurred due to the “always-on” state of the AnV–fluorophore conjugate. This demonstrates that P-IID can be used without a washing step, which is a significant advantage for real-time imaging and high-throughput assays.

Finally, to compare the binding kinetics of P-IID with AnV, flow cytometric profiles of cell populations stained with P-IID or AnV, either at 4°C or with shorter staining times were compared. Staining efficacy remained constant for P-IID with a reduction in temperature from 25°C to 4°C, whereas lowering the temperature greatly reduced the efficacy of AnV staining (Figure 4E, Supporting Figures S9 and S10). Similarly, when the AnV staining time was reduced from 15 min to 5 min or 1 min, distinction between the AnV+ and AnV− population in either CPT-treated MCF-7 cells (Figure 4F, quantification in Figure 4G) or HCC-1806 cells (Supporting Information, Figure S17) became difficult, reflecting the slow binding kinetics of AnV to PS. In contrast, identical staining patterns were observed upon incubating MCF-7 and HCC-1806 cells with P-IID for either 15 min, 5 min, or 1 min. This indicated that staining with P-IID can be performed extremely quickly and without the need for incubation periods, suggesting rapid binding kinetics of P-IID to PS.

Having determined that P-IID binds rapidly to cell surface PS with a fluorogenic response, we assessed the utility of P-IID in real time imaging of cells undergoing apoptosis. Using confocal microscopy, we imaged HCC-1806 cells to which both P-IID (15 μM) and CPT (40 μM) were added under an atmosphere of 5% CO2 at 37°C over the course of 9 h. (Figure 5A). A net fluorescence enhancement in the P-IID channel was observed, with an increase in mean fluorescence intensity (MFI) commencing approximately 3 h after addition of CPT and plateauing after 7 h (Figure 5B). Healthy control cells imaged in the presence of P-IID did not show a fluorescence increase over the same time period (Figure 5C and Supporting Information, Figure S18). These results confirm that P-IID can be used for time-lapse imaging of drug-induced apoptosis in cell populations.

In conclusion, a novel fluorogenic probe for cell surface phosphatidylserine has been synthesized and evaluated. P-IID senses cell surface phosphatidylserine by fluorescence “turn-on” using an intramolecular indicator displacement (IID) mechanism. This is the first time an IID probe has been used in live cells. P-IID detects cell surface phosphatidylserine on apoptotic cells using both confocal microscopy and flow cytometry, and enabled time-lapse imaging of cells undergoing apoptosis. P-IID circumvents some of the major drawbacks associated with AnV by reliably detecting cell surface phosphatidylserine in the absence of Ca2+, at 4°C, and without the need for a wash step. Furthermore, P-IID binding to phosphatidylserine is extremely rapid (1 min vs. 15 min for AnV). P-IID is readily synthesized using a modular approach that will allow the incorporation of alternative fluorophores, and our current studies are focussed on development of a suite of analogues. Taken together, these characteristics make P-IID a powerful tool for imaging cell surface phosphatidylserine. It is superior to fluorescent conjugates of annexin V for the differentiation of living, apoptotic, and necrotic cells in co-staining experiments with propidium iodide.

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Figure 5. A) Time-lapse imaging of a single field of HCC-1806 cells simultaneously treated with CPT (40 μM) and stained with P-IID (15 μM) over the course of 6 h (5% CO2, 37°C). B) Quantification of the change in the mean fluorescence intensity (MFI) over the total imaging time of 9 h. C) Change in MFI when untreated HCC-1806 cells were incubated with P-IID (15 μM) for 9 h. Scale bar: 30 μm. Data are represented as the mean ± SEM from three independent experiments.
Conflict of interest

The authors declare no conflict of interest.

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