A fluorogenic probe for cell surface phosphatidylserine using an intramolecular indicator displacement sensing mechanism


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 such as temperature sensitivity, Ca²⁺ dependence, and slow binding kinetics. Here, we describe a fluorogenic probe for cell surface PS, P-IID, 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. In particular, P-IID binding to cell surfaces is not reliant on cells being at room temperature or the presence of calcium ions, does not require a wash step, and is significantly faster than that of Annexin V. 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 for the first time.

Phosphatidylserine (PS), an anionic phospholipid, is a minor but important component of the membrane of all eukaryotic cells.[1] In healthy cells, PS is almost exclusively found on the inner (cytoplasmic-facing) leaflet of the cell membrane.[2] The exposure of PS on the cell surface is a common marker of cell death[3] and one of the earliest hallmarks of apoptosis (programmed cell death),[4] where externalization of PS acts as a signal for phagocytes to recognize and engulf the dying cells.[5] The ability to selectively and rapidly detect apoptotic cells is crucial across a range of applications in molecular imaging and clinical medicine.[6] 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.[7] The most frequently used probe for detection of PS externalization is fluorescently labeled Annexin V (AnV), a 35 kDa protein,[8] which binds with high affinity to PS in a Ca²⁺-dependent manner.[9] However, there are a number of problems associated with the use of fluorescently labeled AnV derivatives to detect cell surface PS.[10] These include (i) binding of AnV to PS exhibits slow binding kinetics which limit its use in high-throughput drug assays,[11] and must be performed at room temperature, precluding concomitant use of other assay systems such as immunostaining,[12] (ii) the millimolar levels of Ca²⁺ required for AnV binding to PS alter cellular physiology and can result in scramblases translocating more PS to the cell surface,[13] and (iii) currently available AnV-fluorophore conjugates are not ‘turn-on’ probes and therefore their use generally requires a washing step to remove 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, we have developed a fluorogenic small molecule probe capitalizing on intramolecular indicator displacement sensing.

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 by a linker.[14][15] 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 exhibit a fluorescence ‘turn-on’ upon binding to the analyte of interest but fluorogenic probes for imaging apoptosis are scarce.[16][17][18]

Conjugating a fluorophore to the binding site (receptor) of a probe in order 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,[19][20][19][21] in which a fluorescent indicator is covalently attached to the receptor through 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 the ‘turn-on’ of fluorescence.

Herein, we describe a novel ZnDPA based probe, P-IID, for detection of apoptosis by sensing of cell surface PS using a fluorogenic ‘turn-on’ IID mechanism. P-IID incorporates three components on a peptide backbone: a bis(ZnDPA) binding motif for PS,[22] 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,[22] and a stearic acid membrane anchor to reduce cellular uptake of the probe (thereby preventing imaging of PS present on the internal cell membrane).[23] 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). Our results show that the IID sensing mechanism provides high sensitivity and rapid binding kinetics with a ‘turn-on’ fluorescence response. P-IID has significant advantages when compared to the standard AnV assay, allowing...
detection of PS-externalization using both fluorescence microscopy and flow cytometry techniques, without either the need for a washing step or the presence of Ca\(^{2+}\) ions. We also demonstrate the utility of P-IID in the real-time imaging of apoptosis. To the best of our knowledge, this is the first time an IID approach to fluorogenic sensing has been utilized in cellular imaging.

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 SI for details). In the final step of the synthesis, complexation of the purified peptide with two equivalents of Zn(II) led to fluorescence quenching (Figure S9). This was attributed to the coordination of the catechol to one of the ZnDPA arms as indicated by DFT calculations (Figure S24), with the associated deprotonation of the catechol moiety confirmed by mass spectrometry (m/z 723.7886; C\(_{71}\)H\(_{93}\)N\(_{15}\)O\(_{10}\)Zn\(_{2}\)\(^{2+}\)).

We next demonstrated the ability of P-IID to detect PS-rich membranes by vesicle titration experiments. Zwitterionic vesicles composed of 100% POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) and anionic vesicles composed of 50% POPC and 50% POPS (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine) were prepared by standard extrusion techniques. Subsequent titrations of P-IID (10 \(\mu\)M, 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 POPC/POPS vesicles resulted in an approximately 4-fold fluorescence increase at around 530 nm (Figure 2B), attributed to displacement of the coumarin indicator from the ZnDPA binding site by PS.

Having demonstrated that P-IID recognizes PS with a fluorescence ‘turn-on’ 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 \(\mu\)M, 12 h). Following CPT treatment, cells were stained with P-IID (20 \(\mu\)M, 20 min) and a commercial cell stain (SYTO85 Orange, 25 nM, 20 min). Confocal microscopy images showed staining of apoptotic cells by P-IID, while healthy control cells remained unstained (Figure 3). Confocal imaging shows that P-IID localizes to the cell membrane of apoptotic cells. Subsequent experiments in HCC-1806 cells showed that the observed membrane staining pattern was maintained across different cell lines (Figure S21). Analogues of P-IID either lacking the membrane anchor or with a coumarin that does not coordinate to the ZnDPA binding site (and

![Figure 1](image1.png)

**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](image2.png)

**Figure 2.** (A, B) Fluorescence spectra of P-IID (10 \(\mu\)M, 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 of the fluorescence intensity (F.I.) at 530 nm as a function of phospholipid concentration.

![Figure 3](image3.png)

**Figure 3.** Confocal microscopy images of untreated and CPT treated HeLa cells (focused on single cells). Cells were stained simultaneously with SYTO85 Orange (cell stain) and P-IID. Scale bars represent 10 \(\mu\)m.
is therefore always ‘on’) were used as controls and did not show the membrane staining observed with P-IID (Figure S21).

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, as compared to Annexin V, using flow cytometry in HCC-1806 and MCF-7 cells. 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 panels and Figures S13-18, see SI for staining conditions). This is comparable to the commonly employed method of double labeling with AnV and PI.[25] In parallel experiments, cells were stained with commercially obtained AnV-Alexa Fluor 647 conjugate (2.5 μg mL⁻¹) and PI. The flow cytometric profiles of apoptotic cells stained with P-IID resembled those stained with the AnV conjugate (AnV) when the recommended “standard conditions” for AnV staining (15 min, 2.5 mM Ca²⁺, 25 °C, washing step after staining) were used (Figure S13-18), 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, i.e. when labeling of apoptotic cells is required in the absence of Ca²⁺, without washing steps, or at low temperatures (i.e. ‘on ice’), we conducted a range of experiments where we modified the standard conditions accordingly, and quantitatively compared the results to those obtained with AnV (Figure 4A-E for HCC-1806, Figures in SI for MCF-7). 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 (Figures in SI). In contrast, AnV only stained apoptotic cell populations in the presence of Ca²⁺ ions (Figure 4C for HCC-1806, Figure in SI for MCF-7).

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 or whether or not a washing step had been performed (Figure 4D, left panel, for HCC-1806 and Figure in SI for MCF-7). 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 panel), with MCF-7 cells ‘no-wash’ AnV staining showed higher numbers of

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**Figure 4.** (A) Representative flow cytometry analysis of untreated and CPT treated HCC-1806 cells stained with propidium iodide (PI) and P-IID in the presence and absence of 2.5 mM Ca²⁺. (B) Quantification of (A) showing the frequency of CPT treated HCC-1806 cells stained PI+ / P-IID+ (late apoptotic) and PI+ / P-IID+ (early apoptotic) with and without 2.5 mM Ca²⁺. (C) Staining of CPT treated HCC-1806 cells with AnV with and without 2.5 mM Ca²⁺. The frequency of cells stained PI+ / AnV+ (late apoptotic) and PI+ / AnV+ (early apoptotic) is shown. (D, E) Staining of CPT treated HCC-1806 cells with P-IID or AnV (D) with or without a washing step and (E) at 25 °C and at 4 °C. Data for (B-E) are represented as the mean ± SEM from three independent experiments; ns, not significant; *P ≤ 0.05; **P ≤ 0.01 (Student’s t-test). (F) Representative flow cytometry histograms of untreated and CPT treated MCF-7 cells stained with P-IID or AnV for 1, 5, and 15 minutes. (G) Quantification of (F) showing the geometric mean fluorescence intensity (gMFI) of CPT treated cells stained P-IID+ and AnV+ after 1, 5, and 15 minute(s). Data are represented as the mean ± SEM from three independent experiments; ns, not significant; **P ≤ 0.01 (one-way ANOVA).
early apoptotic as well as late apoptotic cells (Figure in SI), 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, in order to compare the binding kinetics of P-IID with AnV, we compared flow cytometric profiles of cell populations stained with P-IID or AnV either at 4 °C or with shorter staining times. We observed that staining efficacy remains constant for P-IID with a reduction in temperature from 25 °C to 4 °C, whereas lowering the temperature greatly reduces the efficacy of AnV staining (Figure 4E, Figures in SI for MCF-7). Similarly, when the AnV staining time was reduced from 15 to 5 or 1 minute(s), distinction between the AnV+ and AnV− population in either CPT treated MCF-7 (Figure 4F, quantification in Figure 4G) or HCC-1806 cells (Figure in SI) became difficult, reflecting the slow binding kinetics of AnV to PS. In contrast, identical staining patterns were observed upon incubating MCF-7 (Figure 4F-G) and HCC-1806 (Figure S20) cells with P-IID for either 15, 5, or 1 minute(s). 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 hours. (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 only P-IID did not show a fluorescence increase over the same period (Figure 5C). These results confirm that P-IID can be used for 'time-lapse' imaging of drug-induced apoptosis in cell populations.

In conclusion, we have synthesized and evaluated a novel fluorogenic probe for cell surface PS. P-IID senses cell surface PS by fluorescence 'turn-on' using an intramolecular indicator AnV by reliably detecting cell surface PS in the absence of Ca2+, at 4 °C, and without the need for a wash step. Furthermore, P-IID binding to PS is extremely rapid (1 min vs. 15 min for AnV). P-IID is readily synthesised using a modular approach that will allow ready 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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Conflict of interest

The authors declare no conflict of interest.

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A fluorogenic probe for cell surface phosphatidylserine enables time-lapse imaging of cell death. The probe also circumvents a number of drawbacks associated with commonly used Annexin V cell death markers (Ca^{2+} dependence, temperature sensitivity, binding kinetics).

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