Biophysical Studies of a Synthetic Mimic of the Apoptosis-Detecting Protein Annexin V

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Abstract. A Zn^{2+}-dipicolylamine coordination compound is shown to sense the presence of anionic phospholipids in a membrane bilayer. The sensor contains two dipicolylamine subunits attached to an anthracene scaffold, which exhibits a maximum absorbance at 380 nm, and undergoes an enhancement in fluorescence intensity when exposed to membranes enriched in phosphatidylserine. For these reasons, the compound is referred to as PSS-380 (**P**hosphatidyl**s**erine **S**ensor, **380** nm). The fluorescence emission of PSS-380 is enhanced up to tenfold by the presence of vesicles containing the anionic phospholipids phosphatidylserine, phosphatidylglycerol, or phosphatidic acid. No enhancement in fluorescence is observed upon exposure to vesicles containing only zwitterionic phosphatidylcholine, or exposure to monodispersed (non-aggregated) anionic phospholipids. The sensing effect is cooperative; not only does association to the vesicles increase if the vesicles have raised levels of anionic phospholipid, but the maximum fluorescence at sensor saturation is also enhanced. It appears that sensing is triggered by the three-component self-assembly of sensor, Zn^{2+}, and the anionic membrane surface, which leads to diminished photo-induced electron transfer (PET) quenching. The utility of PSS-380 in flow cytometry and fluorescence microscopy is demonstrated by using the molecule to detect the appearance of phosphatidylserine on the plasma membrane surface of various cell lines. Thus, PSS-380 can identify apoptotic cells in the same way as the commonly used protein reagent annexin V.

INTRODUCTION

The asymmetric transmembrane distribution of phospholipids is a fundamental feature of normal cell operation. In the case of animal plasma membranes, most of the phosphatidylethanolamine (PE) and phosphatidylserine (PS) is sequestered in the inner monolayer of the membrane, whereas most of the phosphatidylcholine (PC) and sphingomyelin (SM) is in the membrane outer monolayer.[1] This organization of lipids is generated and maintained by the concerted action of a number of phospholipid translocases that vary in lipid specificity, energy requirements, and direction of translocation.[2] The PS localized in the inner monolayer is vital for exocytosis and intracellular fusion processes, as well as lipid–protein interactions and signal transduction pathways.[3–5] When cells become apoptotic, the ensuing morphological and biochemical processes can be monitored in order to quantify the level of apoptosis in a cell population. These events include movement of PS to the outer leaflet of the cell membrane, breakdown of nucleic acids, upregulation of cytosolic caspase activity, and eventual membrane permeabilization. Each of these cellular changes can be used as an independent apoptosis assay; however, the appearance of PS on the

Abbreviations: 7AAD, 7-aminoactinomycin D; AZD, anthracene zinc-dipicolylamine; DHPC, dihexanoylphosphatidylcholine; DHPS, dihexanoylphosphatidylserine; EGTA, ethyleneglycol tetraacetic acid; PC, phosphatidylcholine; PI, propidium iodide; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; PS, phosphatidylserine; TES, N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid.

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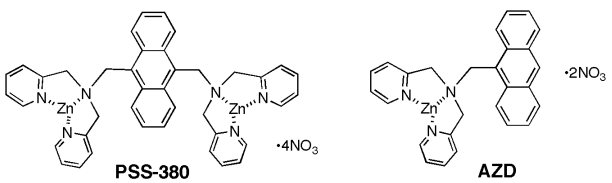
cell membrane surface is the best indicator of the early to intermediate stages of the process.[6–11] Flow cytometry and fluorescence microscopy can detect apoptotic cells if they are stained with a fluorescently-labeled reagent that selectively recognizes the externalized PS. A frequently used reagent for such PS recognition is the membrane-binding protein annexin V.[10–14] Various labeled versions of this 35-kDa protein are commercially available, although annexin V–fluorescein conjugates are the most popular as they are compatible with commonly used flow cytometers. The high value of this protein reagent is without doubt, but it is not without limitation. Firstly, annexin V–membrane binding is calcium dependent and requires up to 2.5 mM Ca^{2+} for complete binding.[11] Such high levels can be problematic because many animal cell membranes also have Ca^{2+}-dependent phospholipid scramblases that can move PS to the extracellular membrane leaflet and lead to false positive results.[14] Furthermore, phospholipid peroxidation is known to increase annexin V membrane affinity,[15] and the kinetics of annexin V–membrane binding can be too slow for certain types of kinetic assays.[16–17] In summary, there is a need for small, non-protein reagents that recognize PS with the same specificity and affinity as annexin V.[18–19]

Recently we reported that the Zn^{2+}-dipicolylamine coordination compound PSS-380 (phosphatidylserine sensor, 380 nm) can be used as a sensor for membranes containing PS, and that this sensing function allows PSS-380 to identify Jurkat cells in the early stages of apoptosis.[20] Subsequently, PSS-380 has been shown by other research groups to be useful for detection of apoptosis in different cell lines,[21] as well as monitoring PS levels in other cellular processes.[22] Here we provide a detailed biophysical study of the binding properties of PSS-380, including further demonstration of its utility as a sensor for apoptosis.



EXPERIMENTAL

Vesicle and Heparin Studies

Unilamellar vesicles were prepared by initial hydration of a dried lipid film followed by repeated extrusion through polycarbonate membranes having a pore size of 100 nm. Aliquots of a vesicle suspension were added to a solution of PSS-380 (1 μM, 3 mL) in HEPES buffer (10 mM) at pH 7.2. Immediately after the addition of each aliquot, the fluorescence emission intensity was recorded at 440 nm (ex 380 nm). Control experiments showed that PSS-380 (a) induces some aggregation of POPS:POPC (1:1) vesicles at low phospholipid to PSS-380 ratios; (b) does not induce leakage of carboxyfluorescein from POPS:POPC (1:1) vesicles at low or high phospholipid to PSS-380 ratios; and (c) does not promote the flip-flop of fluorescently labeled NBD-phospholipid probes. Heparin was obtained from Sigma and used without further purification. Aliquots of a 1.0 mM aqueous heparin solution were sequentially added to a 1 μM solution of PSS-380 until the final concentration of heparin reached 100 μM. No change in fluorescence intensity was observed.

Erythrocyte Studies

Aliquots (10 mL) containing 4.0×10^9 normal or "scrambled" erythrocytes were added to a solution of PSS-380 (1 μM, 3 mL) in 5 mM TES, 150 mM NaCl, pH 7.4. The erythrocytes were prepared in the following way.[14] Fresh human blood was collected in ice-cold phosphate-buffered saline (PBS) containing 10 U/mL of heparin. The samples were centrifuged, and the supernatant and the top 10% of the erythrocytes were removed. The wash was repeated two more times. An equal volume of PBS was added to the remaining pellet. The desired amount of suspension was removed and centrifuged, and the supernatant discarded. Four volumes of lysis buffer (0.1 × PBS containing 0.1 mM EGTA, 1 mM $MgCl_2$, and 1% BSA, plus 1 mM $CaCl_2$ for "scrambled" erythrocytes only) were added to the pellet. The suspensions were vortexed and the lysed cells were placed on ice. After 2 min, the cells were resealed by adding 0.4 volumes (relative to the size of the original pellet) of 10 × PBS. The tubes were gently inverted and warmed to 37 °C. After 30 min, 100 volumes of PBS were added, then the cells were centrifuged into pellets, washed three times, and resuspended in TES buffer for use.

Cell Staining and Fluorescence Microscopy

Annexin V-FITC (Pharmingen) and 7AAD (Sigma) were obtained commercially, whereas PSS-380 and AZD were synthesized.[23] Jurkat E6-1 cells were obtained from American Type Culture Collection and grown in RPMI 1640 growth media supplemented with 10% fetal bovine serum. MCF-7 cells, provided by Professor J. Welsh, University of Notre Dame, were grown in DMEM growth media with 10% fetal bovine serum. These adherent cells were grown to 80% confluence directly in 16-well chamber microscope slides. Suspended cells (Jurkat) were grown to a density of 1×10^6 cells/mL. Before staining with annexin V-FITC, the growth media was changed to a Binding Buffer containing 5 mM TES/145 mM NaCl, 2.5 mM $CaCl_2$, at pH 7.4. The cells were incubated with annexin V-FITC (10 μL of Pharmingen stock solution per milliliter cell suspension), 7AAD (5 μL of 50 mg/mL stock solution per milliliter cell suspension), and PSS-380 (20 μL of 1 mM stock solution per milliliter cell suspension) for 15 min at 37 °C. The incubation mixture was aspirated and changed to a buffer containing 5 mM TES, 145 mM NaCl, pH 7.4. At this point, the Jurkat cell suspensions were added to 16-well chamber-slides. Cells were observed directly on an Axiovert S100 TV microscope (Carl Zeiss) equipped with filter sets DAPI/Hoechst/AMCA, FITC/

RSGFP/Bodipy/Fluo3/DiO, Cy3 (Chroma). Pictures were taken using a black and white digital camera and colored afterwards using Photoshop 6.0 software package (Adobe).

Flow Cytometry

HL60 cells were cultured in RPMI 1640 medium and removed after overnight expansion. Apoptosis was induced by treatment with camptothecin (10 μM) for 6 h. Before staining, the cells were washed twice (300*g*, 10 min) in cold Earle's Balanced Salt Solution followed by resuspension in Binding Buffer to a concentration of ~1 × 10^6 cells/mL. Aliquots (100 μL) of this cell suspension were transferred to 12 × 75 mm test tubes, along with appropriate amounts of annexinV-FITC (5 μL of Pharmingen stock solution), PSS-380 (10 μL of 1 mM stock solution), and PI (10 μL of 50 mg/mL stock solution). The tubes were gently agitated for 15 min at room temperature in the dark, then an additional 400 mL of Binding Buffer was added. Another incubation for 10 min was followed by a wash (300*g*, 10 min) and resuspension in 400 mL of Earle's Buffer prior to flow cytometry. All tubes were analyzed within 1 hour of preparation. Flow cytometry was performed using a Coulter Altra flow cytometer equipped with an Enterprise II laser.[24] PSS-380 was excited at 350 nm and emission was collected at 440 nm, annexin V was excited at 488 nm with emission at 525 nm, and PI was excited at 488 nm with emission at 617 nm. The cell analysis was gated to allow counting of only PI-negative cells.

RESULTS

The change in fluorescence emission for PSS-380 upon addition of POPC:POPS (50:50) vesicles is shown in Fig. 1. Plots of change in fluorescence intensity upon addition of vesicles composed of 100% POPC, and 50:50 mixtures of POPC:POPS, POPC:POPG, and POPC:POPA are shown in Fig. 2. No change in the fluorescence of PSS-380 was observed after addition of zwitterionic POPC vesicles. However, fluorescence intensity did increase if the vesicles contained an anionic phospholipid in the order POPG (11-fold increase) > POPS (10-fold increase) > POPA (6-fold increase). Even vesicles containing 5 mol% POPS produced a significant increase in fluorescence (Fig. 3). Further-

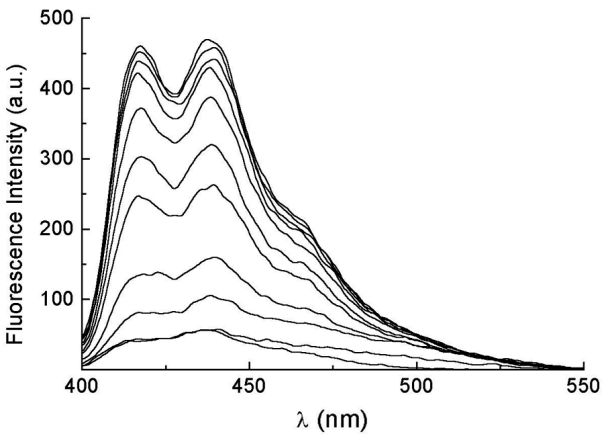


Fig. 1. Fluorescence emission (ex 380 nm) of PSS-380 (1 μM) in HEPES buffer (10 mM, pH 7.2) upon addition of 1 to 10 mM (total phospholipid concentration) of POPC:POPS (50:50) vesicles. a.u. = arbitrary units.

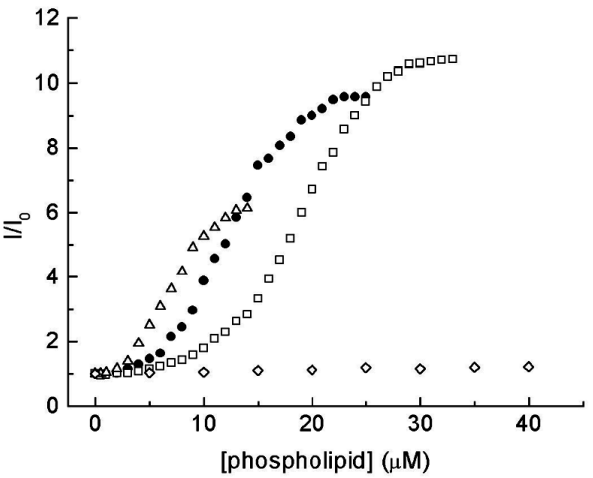


Fig. 2. Change in fluorescence intensity I/I_o (ex 380, em 440 nm) of PSS-380 (1 μM) in HEPES buffer (10 mM, pH 7.2) upon addition of 100-nm unilamellar vesicles composed of 100% POPC (diamonds), or 50:50 mixtures of POPC:POPS (filled circles), POPC:POPG (squares), or POPC:POPA (triangles).

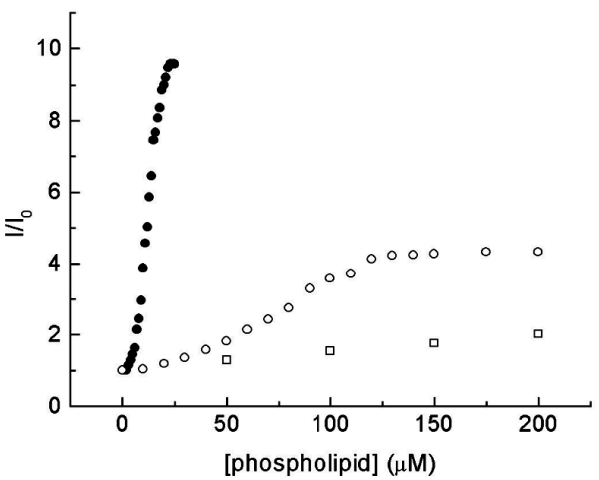


Fig. 3. Change in fluorescence intensity I/I_o (ex 380, em 440 nm) of PSS-380 (1 μM) in HEPES buffer (10 mM, pH 7.2) upon addition of 100-nm unilamellar vesicles composed of POPC:POPS 50:50 (filled circles), POPC:POPS 95:5 (open circles), 100% POPC (squares).

more, the sensing effect is cooperative; not only does binding to the vesicles increase with raised PS levels, but the maximum fluorescence at vesicle saturation is also enhanced. Interestingly, no fluorescence enhancement was observed when PSS-380 was treated with up to a 100-fold excess of heparin (data not shown).

Although the fluorescence emission of PSS-380 increases in the presence of vesicles containing PS, it is unchanged if the PS is monodispersed (non-aggregated). For example, DHPC and DHPS are PC and PS analogues with short C_6-acyl chains, and therefore do not aggregate at micromolar concentrations.[25] Titration of PSS-380 with increasing amounts of a DHPC:DHPS (1:1) mixture results in no change in fluorescence intensity (Fig. 4). Further evidence that PSS-380 has only a weak affinity for DHPS and DHPC in water was gained from ^{31}P NMR titration experiments. The ^{31}P signals for phospholipid head groups hardly move upon addition of PSS-380. This is in line with previous studies with PSS-380 showing that it has low affinity for neutral or monoanionic molecules in water.[23] Both of the zinc dipicolylamine groups in PSS-380 are necessary for the efficient detection of PS-enriched vesicles because treatment of AZD, a control analogue that lacks one of the zinc dipicolylamine groups,[23] with excess POPC:POPS (50:50) vesicles produces very little enhancement in fluorescence (Fig. 5).

A major goal of this project is to examine the behavior of PSS-380 in cellular systems. First, we evaluated the ability of PSS-380 to detect the presence of PS on the surface of erythrocytes, a relatively simple cell system that normally maintains most of its PS in the inner monolayer of its plasma membrane. Using a standard procedure, we prepared "scrambled" erythrocytes with a symmetrical distribution of PS on both sides of the plasma membrane.[14] As shown in Fig. 6, the "scrambled" erythrocytes induce a much higher PSS-380 fluorescence, compared to an equal number of normal erythrocytes, which is attributed to their higher levels of externalized PS.

We turned next to the question of whether PSS-380 could be used to classify cells as either live, apoptotic, or dead. To answer this question we used fluorescence

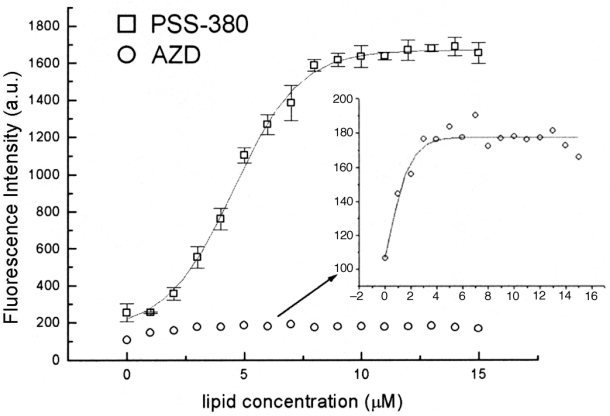


Fig. 5. Change in fluorescence intensity (ex 380, em 440 nm) of PSS-380 (1 μM) in HEPES buffer (pH 7.2, 10 mM) upon addition of 100-nm unilamellar vesicles composed of varying ratios of POPC:POPS (50:50). Comparison with AZD. The inset is an explosion of the fluorescence emission behavior of AZD represented on a different vertical scale from the full figure in order to clearly illustrate the small change in fluorescence emission intensity.

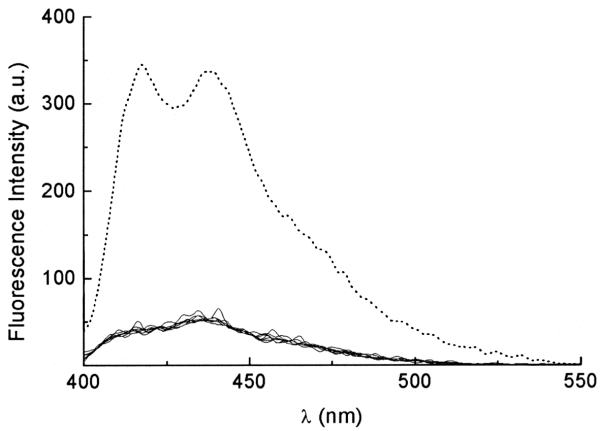


Fig. 4. Fluorescence emission of PSS-380 (1 μM) in HEPES buffer (10 mM, pH 7.2) upon addition of 10 mM (total lipid concentration) of POPC/POPS (50:50) vesicles—dotted line, or 1 to 10 mM (total lipid concentration) of DHPC/DHPS (50:50)—solid lines. l_{ex} = 380 nm.

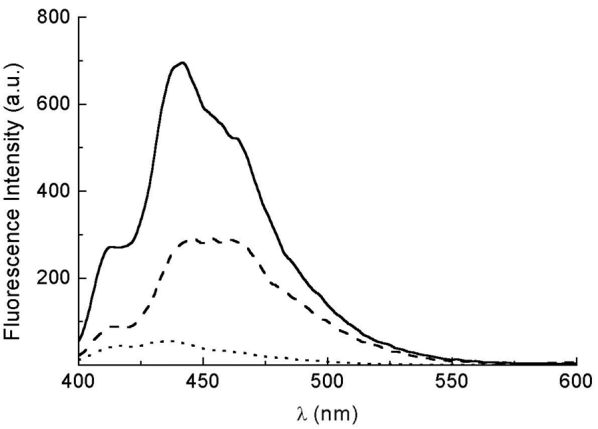


Fig. 6. Fluorescence emission of PSS-380 (1 μM) in 5 mM TES, 150 mM NaCl, pH 7.4 upon excitation at 380 nm (dotted line), presence of normal erythrocytes (dashed line), or same number of symmetrical "scrambled" erythrocytes (solid line).

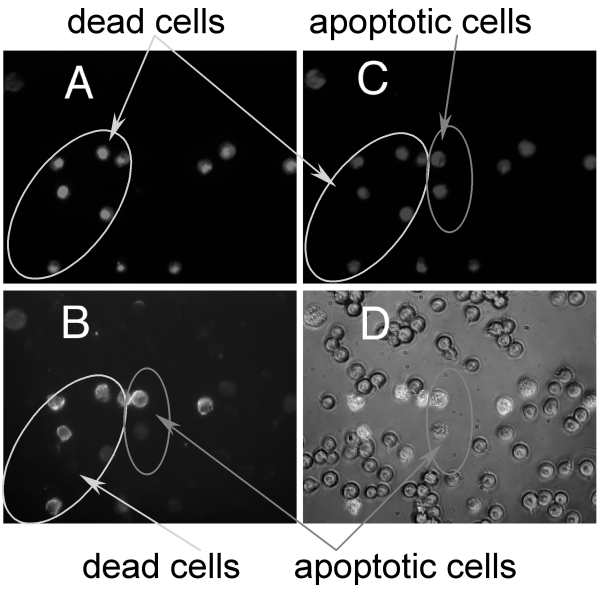


Fig. 7. Apoptosis of Jurkat cells induced by incubation with calcium ionophore A23187 (0.2 μM) for 30 min. The cells were stained simultaneously with 7AAD (A), annexinV-FITC (B), PSS-380 (C). (D) is a merge of (A), (B), (C), and a phase contrast image of the same cells. Live cells are not stained by any of the dyes.

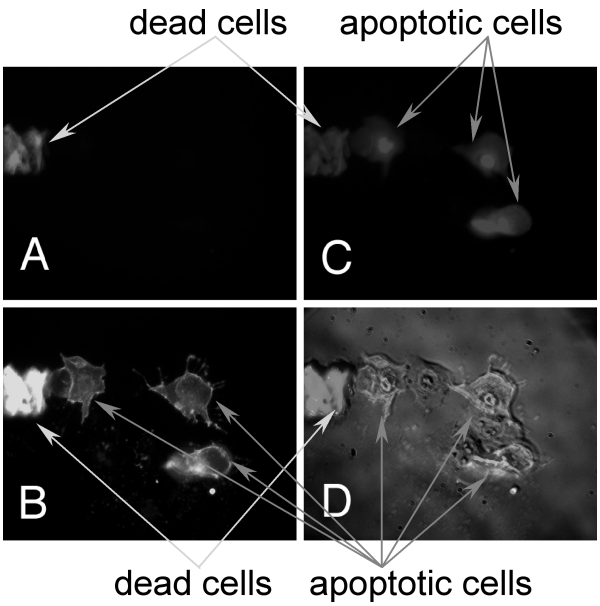


Fig. 8. Apoptosis of MCF-7 cells induced by incubation with calcium ionophore A23187 (2.5 μM) for 15 min. The cells were stained simultaneously with 7AAD (A), annexinV-FITC (B), PSS-380 (C). (D) is a merge of (A), (B), (C), and a phase contrast image of the same cells. Live cells are not stained by any of the dyes.

microscopy to observe Jurkat cells, either untreated, or treated with camptothecin or the calcium ionophore A23187 to induce apoptosis. All samples were stained with PSS-380 (blue emission), annexin V-FITC (green emission), or the nuclear stain 7AAD (red emission). Figures 7 and 8 display typical microscopic images produced using this method. Live cells are not stained by any of the dyes, while staining by 7AAD identifies dead cells with permeable membranes. Cells that are only stained by annexinV-FITC or PSS-380 are defined as apoptotic. The microscopic images show the expected statistical variation in cell staining, which is further complicated by the fact that PSS-380 and annexin V-FITC are competing for the same cell surface binding sites. If the staining experiments are repeated using either annexin V or PSS-380 alone, the fluorescence emission is more homogeneous and with similar intensity for all positive cells. Competition studies show that pretreatment of the apoptotic cells with PSS-380 blocks staining by annexin V-FITC in a dose-dependent manner, indicating that both reagents are binding to the same site.

The ability of PSS-380 to detect apoptotic cells is further demonstrated by the flow cytometry data in Fig. 9, which measure the fraction of apoptotic HL60 cells generated after treatment with camptothecin. The cell population shown in Fig. 9 indicates that 80–85% of the viable cells (determined by exclusion of the nuclear stain propidium iodide) are stained by PSS-380 and can be classified as apoptotic. The same fraction was obtained by staining a separate sample of the same cell population with annexin V-FITC (data not shown).

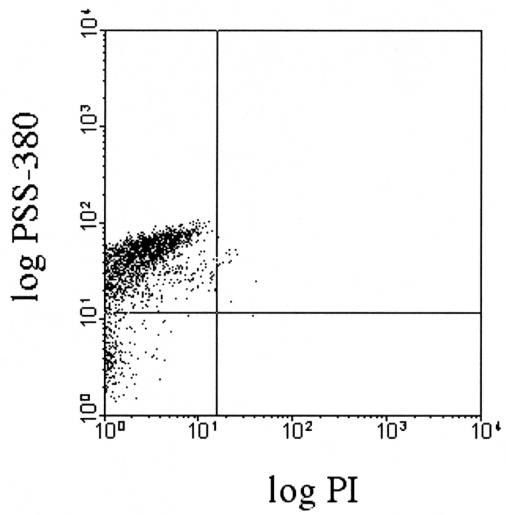
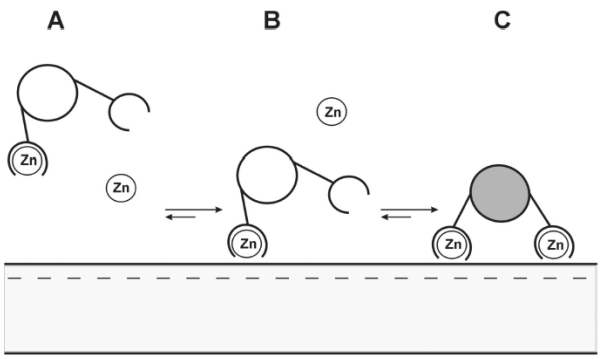


Fig. 9. Flow cytometry analysis showing that treatment of HL60 cells with camptothecin (10 μM) for 6 h induces cell apoptosis and staining by PSS-380. PI is propedium iodide.

DISCUSSION

The fluorescence of PSS-380 in water is apparently not affected when treated with monodispersed anionic phospholipids. However, a large increase in fluorescence is observed if PSS-380 interacts with vesicles containing anionic phospholipids such as PS. The sensing effect is cooperative; not only does association to the vesicles increase if the vesicles have raised levels of PS, but the maximum fluorescence at sensor saturation is also enhanced. This indicates that binding of the cationic PSS-380 to membrane-bound PS is stronger and gives a much higher fluorescence than binding to membrane-bound PC. In addition, a control compound, AZD, with one zinc dipicolylamine group, exhibits no fluorescence enhancement when it is treated with excess PS-rich vesicles. Together, the results suggest that fluorescence enhancement is not simply due to a membrane-induced change in polarity surrounding the PSS-380 fluorophore. Previous studies by Hamachi and coworkers[26] have shown that the first Zn^{2+} is bound by PSS-380 with an association constant of approximately 10^7 M^{-1}, and the second Zn^{2+} exhibits an association constant of only around 10^4 M^{-1}. This means that at a concentration of 1 μM, the predominant coordination complex in solution is the mono-zinc species A in Scheme 1. In this form, the lone pair electrons on the nitrogen of the second uncomplexed dipicolylamine moiety are able to quench the anthracene fluorescence by photo-induced electron transfer (PET). Binding of the mono-zinc species to an anionic membrane surface (B, Scheme 1) appears to promote a three-component assembly process, where the sensor, the membrane, and additional Zn^{2+} adsorbed onto the membrane surface combine to form the bis-zinc species (C, Scheme 1). In this fully coordinated form, the nitrogen lone pair electrons in both dipicolylamine systems are unable to quench by PET; therefore a fluorescence enhancement is observed when PSS-380 interacts with an anionic membrane surface. This binding model is very similar to that used by annexin V, where the three-component assembly process involves the protein, Ca^{2+}, and the anionic membrane surface.



Scheme 1. Binding of PSS-380 to the surface of a membrane containing anionic phospholipids. The net negative charge on the membrane surface is denoted by the multiple minus signs (–) shown above.

The flow cytometry data indicates that PSS-380 and annexin V are binding to the same population of apoptotic cells. It is worth highlighting other functional similarities and differences between PSS-380 and annexin V. Both reagents associate selectively with membrane surfaces containing anionic phospholipids.[27–28] The association is driven primarily by electrostatic interactions with the bridging metal cations (Zn^{2+} in the case of PSS-380 and Ca^{2+} in the case of annexin V), and augmented with weaker protein–phospholipid interactions in the case of annexin V.[29] The microscopy data indicate that PSS-380 staining is more intense than annexin V, which is attributed to annexin V's much larger size per fluorophore. It has been estimated that annexin V (molecular weight of 35 kDa) covers a surface binding area of about 50 phospholipid molecules.[27] Thus, it is possible that more than ten PSS-380 molecules (molecular weight of 1 kDa) can bind to the same membrane area as one annexin V molecule.

In addition to its utility in cell biology research, PSS-380 may also find application in the automation of biotechnology processes[13] and high-throughput screening systems for drug candidates.[30] It should be noted that even though PSS-380 alleviates many technical drawbacks of annexin V, its use is limited by the anthracene fluorophore, which is presently incompatible with many commercially available flow cytometers. We are currently pursuing analogues of PSS-380 that incorporate reporter elements more suitable for common laboratory instrumentation. From a broader perspective, we demonstrate here that a small-molecule Zn^{2+}-dipicolylamine coordination compound can serve as a functional mimic of a large protein, annexin V. It seems reasonable to expect that similar zinc coordination compounds will mimic other supramolecular functions of the annexin family of proteins, which play key regulatory roles in a number of cellular processes.[31–32]

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