CHEMBIOCHEM

DOI: 10.1002/cbic.200500149

Fluorescent Detection of Apoptotic Cells by Using Zinc Coordination Complexes with a Selective Affinity for Membrane Surfaces Enriched with Phosphatidylserine

Roger G. Hanshaw,[a] C. Lakshmi,[a] Timothy N. Lambert,[b] James R. Johnson,[a] and Bradley D. Smith*[a]

The appearance of phosphatidylserine on the membrane surface of apoptotic cells (Jurkat, CHO, HeLa) is monitored by using a family of bis(Zn^{2+}-2,2′-dipicolylamine) coordination compounds with appended fluorescein or biotin groups as reporter elements. The phosphatidylserine affinity group is also conjugated directly to a CdSe/CdS quantum dot to produce a probe suitable for prolonged observation without photobleaching. Apoptosis can be detected under a wide variety of conditions, including variations in temperature, incubation time, and binding media. Binding of each probe appears to be restricted to the cell membrane exterior, because no staining of organelles or internal membranes is observed.

Introduction

An important structural feature of healthy animal cells is the asymmetric distribution of phospholipids between the two leaflets of the cell plasma membrane.[1] In particular, phosphatidylserine (PS), which usually constitutes less than 10% of the total phospholipid in the membrane, is confined almost exclusively to the inner monolayer.[2] During the early stages of programmed cell death (apoptosis), the phospholipid distribution is scrambled, which leads to the appearance of PS on the cell surface. Thus, the detection of cells with externalized PS is a straightforward and widely used assay for apoptosis.[3–9] The assay usually employs annexin V, a 36 kDa protein with a calcium-dependent affinity for membranes that are enriched in anionic phospholipids.[10] A range of annexin V–dye conjugates is now commercially available and used extensively in cell-biology research. However, annexin V has some drawbacks that diminish its utility in certain applications. For example, the protein might not have the necessary chemical stability for employment in high-throughput screening of cancer drugs[11] and can lack the biochemical stability necessary for in vivo imaging of dying tissue.[12–13] Other potential problems are the slow kinetics of membrane binding[14] and the calcium dependence, which can potentially lead to artifacts such as the activation of nonspecific membrane scramblase activity.[15] It is apparent that a small-molecule substitute for annexin V that binds PS-rich membranes in a Ca^{2+}-independent manner would be a very useful reagent for detecting apoptosis.

The idea that a small molecule can be used to detect apoptosis is supported by work with the fluorescent dyes MC 540[16] and FM1-43.[17] The binding of MC 540 to membranes is sensitive to membrane composition, and binding increases when cells become apoptotic. However, there are significant disadvantages with MC 540. The signal difference between normal cells and apoptotic cells is only about fivefold (it is up to 100-fold with annexin V); furthermore, the dye is phototoxic. Likewise, FM1-43 is a cationic dye that binds more tightly to membranes that are rich in anionic phospholipids and gives a signal difference between normal and apoptotic cells of six- to tenfold. Another disadvantage with FM1-43 is its broad emission (515–595 nm), which makes it difficult to use a second fluorophore that emits in the fluorescein or rhodamine channels. Cationic vesicles[18] and nanoparticles coated with cationic peptide sequences[19] have also been reported to bind to apoptotic cells, but the practicality of these agents in apoptosis assays is likely to be limited.

Recently, we discovered that fluorescent Zn^{2+}-dipicolylamine (DPA) coordination complexes have a selective affinity for membrane surfaces that are enriched in anionic PS, and that they can be used to detect apoptosis.[20] Our initial studies used fluorophores with nonoptimal properties,[21] but the work produced important structural information that led us to the modular, three-component design shown in Scheme 1. The

[a] *R. G. Hanshaw, Dr. C. Lakshmi, J. R. Johnson, Prof. Dr. B. D. Smith*
Department of Chemistry and Biochemistry and
Walther Center for Cancer Research, University of Notre Dame
Notre Dame, IN 46556 (USA)
Fax: (+1) 574-631-6652
E-mail: smith.115@nd.edu

[b] *Dr. T. N. Lambert*
Chemical Synthesis and Nanomaterials, Sandia National Laboratories
Albuquerque, NM 87185 (USA)

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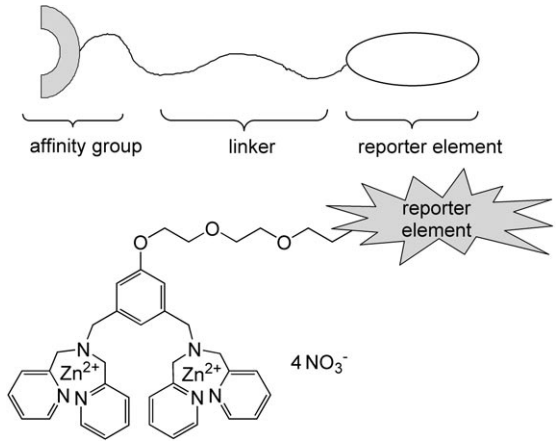
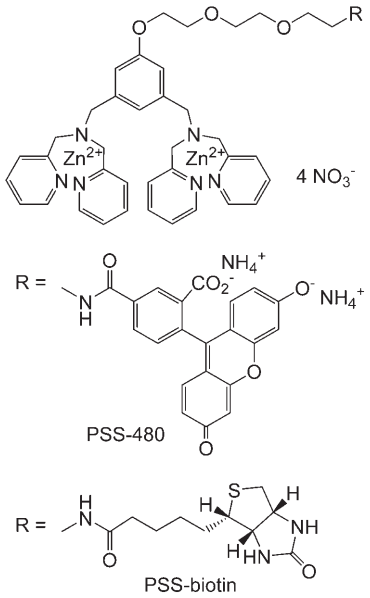


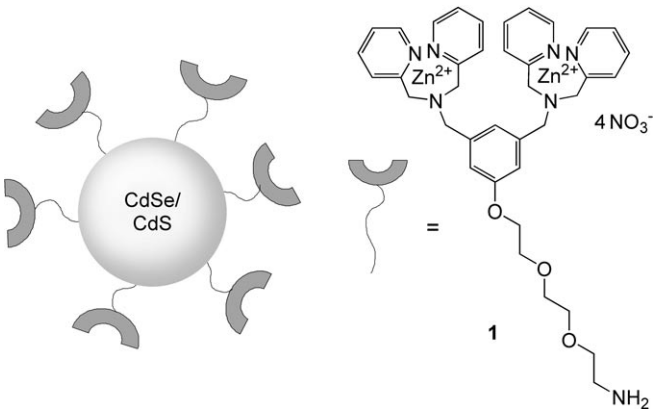
Scheme 1.

design contains a PS affinity group (two Zn^{2+}–DPA subunits that are *meta* oriented on a phenyl ring) that is attached via a tris(ethyleneoxy) linker to a reporter element. In this report we describe some examples of this design that incorporate practical reporter elements (Scheme 2). Specifically, we evaluate the



Scheme 2.

utility of the fluorescein derivative PSS-480 (*p*hosphatidyl*s*erine *s*ensor, *480* nm excitation) in fluorescence-microscopy and flow-cytometry experiments. We have also prepared PSS-biotin, a biotinylated version that can be visualized by using indicator-labeled streptavidin, including streptavidin-conjugated quantum dots. Finally, we describe a CdSe/CdS quantum-dot system (PSS-green QD) coated with the PS affinity group (Scheme 3).



Scheme 3.

Results

Synthesis

The fluorescein-labeled compound PSS-480 was prepared by coupling the known amine **1**[21] with 5-carboxyfluorescein diacetate. Subsequent deprotection with ammonium hydroxide followed by treatment with $Zn(NO_3)_2$ in aqueous methanol gave PSS-480 as its bis(ammonium) salt.

The biotin-linked compound PSS-biotin was produced by coupling **1** and biotin, followed by complexation with $Zn(NO_3)_2$ in aqueous methanol. The PEG-micelle encapsulated quantum dots started with CdSe/CdS core/shell nanocrystals that were prepared by following existing literature methods.[22–24] Transmission electron microscopy showed that the quantum dots were of uniform diameter (~4–5 nm) and not aggregated (Figure 1). These organic soluble nanoparticles were subsequently encapsulated in carboxy-PEG–phospholipid micelles;[24] this rendered them water soluble. The exposed carboxyl residues were coupled with the bis-Zn^{2+} complex of amine **1** to

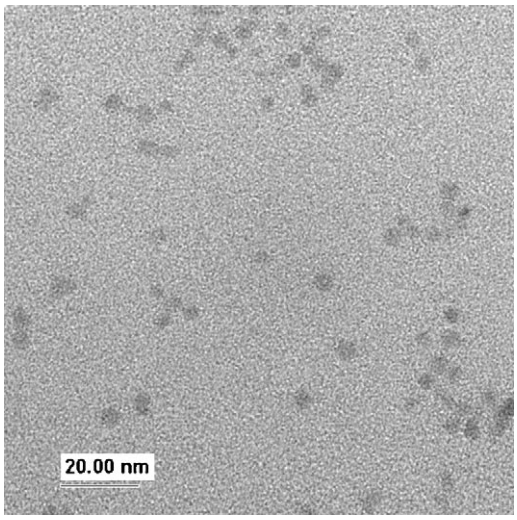


Figure 1. Transmission electron micrograph of CdSe/CdS nanoparticles used to prepare the PSS-green QD. The sample was prepared by allowing a solution of nanoparticles in $CHCl_3$ to evaporate on a copper-coated TEM grid. The particles exhibit a roughly spherical geometry of 4–5 nm in diameter.

give Zn^{2+}–DPA-conjugated micelle encapsulated quantum dots (PSS-green QD) (Scheme 3).

Fluorescence microscopy

Fluorescence microscopy was used to determine if PSS-480, PSS-biotin, and PSS-green QD could selectively stain apoptotic cells. Jurkat cells were treated first with the anticancer drug camptothecin to induce apoptosis, and then simultaneously with PSS-480 and the nuclear stain 7AAD (7-aminoactinomycin D; Figure 2). Necrotic cells, as well as those cells in the advanced stages of apoptosis, have permeabilized membranes and allow 7AAD to stain the nucleic acid. Healthy cells and those cells in the early-to-intermediate stages of apoptosis retain their membrane integrity and exclude 7AAD. This allows cells in early apoptosis to be identified by selective staining with PSS-480 and exclusion of 7AAD. A bright-field image of a field of cells stained with PSS-480 (Figure 2D) clearly illustrates that the PS affinity group binds only to those cells with externalized PS. Similarly, PSS-480 was used to identify HeLa and CHO cells exposing PS on the membrane surface (see Supporting Information).

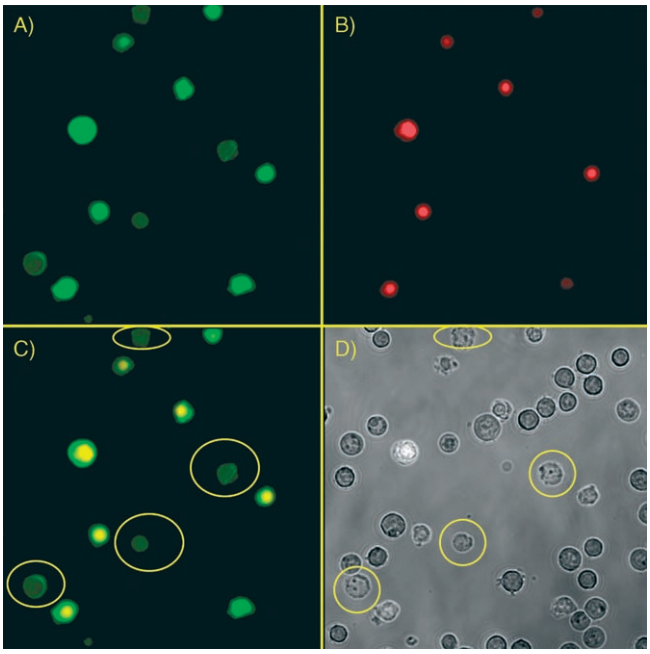


Figure 2. Fluorescence micrographs (40× magnification) of Jurkat cells treated with camptothecin (10 μM) for 3.5 h to induce apoptosis, then stained with PSS-480 (5 μM) and 7AAD (500 ng mL^{-1}). A) Fluorescence of cells stained with PSS-480; B) fluorescence of cells stained with 7AAD; C) an overlay of (A) and (B). Those cells stained only with PSS-480 are apoptotic, as illustrated by exclusion of 7AAD. The apoptotic cells are indicated with circles. D) A phase-contrast image of all cells in the field. No staining of healthy cells with PSS-480 was observed in the absence of treatment with camptothecin.

Evidence that the PS-affinity group is binding to the same membrane sites as annexin V is provided in Figure 3, which illustrates the co-staining of apoptotic Jurkat cells with 7AAD, annexin V-FITC (fluorescein isothiocyanate), and the PSS-biotin, visualized by using a blue-emitting streptavidin conjugate. The circled cells are apoptotic, as evidenced by their staining only with annexin V and PSS-biotin/streptavidin with simultaneous exclusion of 7AAD. Additional co-staining evidence is supplied in Figure 4, which shows fluorescent cross sections of a single Jurkat cell treated with annexin V-

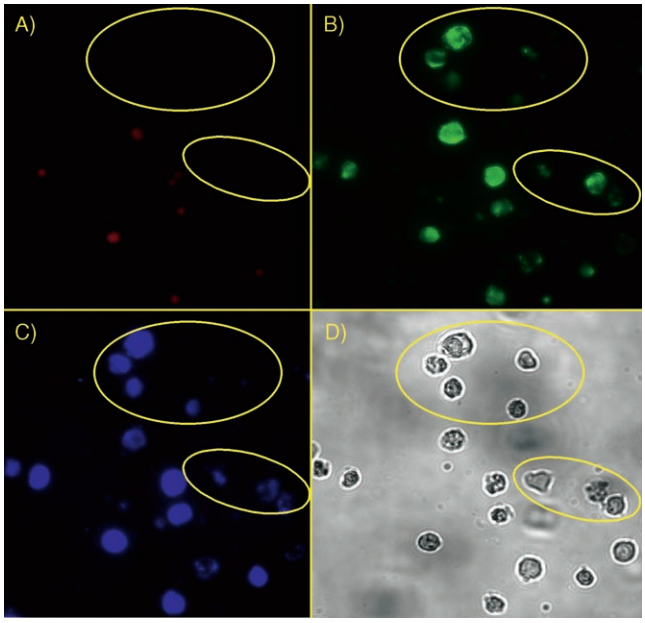


Figure 3. Fluorescence micrographs (40× magnification) of Jurkat cells treated with 10 μM camptothecin for 3.5 h and stained with 7AAD (500 ng mL^{-1}), annexin V-FITC, and PSS-biotin (100 μM) with a blue-emitting streptavidin–dye conjugate (400 nM). All reagents were added simultaneously. Cells were then incubated for 15 min at 37 °C. A) Cells stained with the nuclear stain 7AAD; B) Cells stained with annexin V-FITC; C) Cells stained with PSS-biotin/streptavidin–Marina Blue conjugate (460 nm emission); D) Bright-field image of the entire field of cells. Cells in the circled regions of each image are apoptotic. No staining of healthy cells was observed in the absence of treatment with camptothecin.

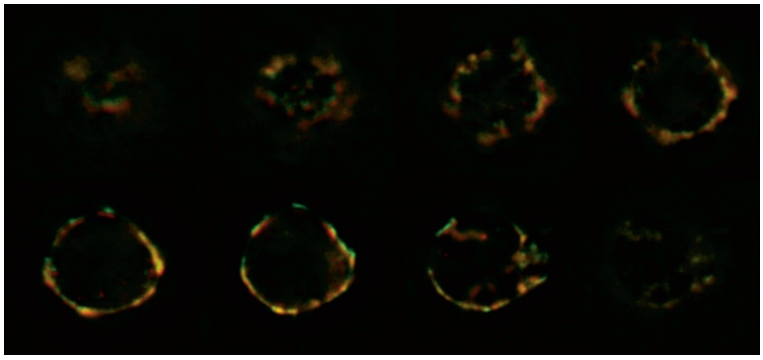


Figure 4. Cell-surface labeling of a Jurkat cell treated with 10 μM camptothecin for 3.5 h to induce apoptosis. Images are 0.5 μm slices (60× magnification) taken through the cell separated by 2.5 μm. Cells were treated with annexin V-FITC and PSS-biotin (100 μM) with a red-emitting (605 nm) streptavidin–quantum dot conjugate (10 nM). The yellow–orange color is a result of annexin V–PSS-biotin/streptavidin–quantum dot colocalization.

FITC and PSS-biotin, and visualized with a red-emitting streptavidin–quantum dot conjugate. The staining is clearly restricted to the exterior cell membrane, and the yellow–orange color signifies colocalization. Selective staining of cells with externalized PS was also observed with PSS-green QD (Figure 5). Cross-sectional micrographs showed that the staining was restricted to the exterior membrane (see Supporting Information). Control experiments indicated that quantum dots coated with ammonium groups instead of the PS-affinity groups do not stain apoptotic cells.

Flow cytometry

The utility of PSS-480 in flow cytometry was demonstrated by using a population of Jurkat cells treated with camptothecin (10 μM, 16 h) to induce apoptosis. The graphs in Figure 6 indicate that approximately 30% of the cells were stained with PSS-480, while less than 5% of the cells were stained by 7AAD (the necrotic subpopulation). With untreated cells, less than 5% of the total population was stained with PSS-480 and 7AAD. Staining of identical cells with 7AAD and annexin V-FITC resulted in essentially the same distribution of subpopulations.

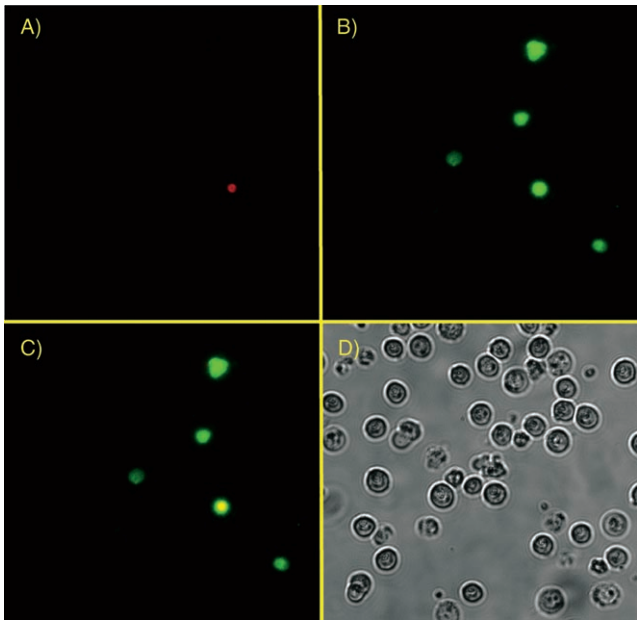


Figure 5. Fluorescence micrographs (40× magnification) of Jurkat cells treated with camptothecin (10 μM) for 3.5 h to induce apoptosis, then stained with PSS-green QD (2.75 μM) and 7AAD (500 ng mL^{-1}). A) Fluorescence of cells stained with 7AAD; B) fluorescence of cells stained with PSS-green QD; C) an overlay of (A) and (B). Those cells stained only with PSS-green QD are apoptotic, as illustrated by exclusion of 7AAD. D) A phase-contrast image of all cells in the field. No staining of healthy cells was observed in the absence of treatment with camptothecin.

Discussion

During apoptosis, the electrostatic charge on a cell-membrane surface becomes increasingly negative as the PS normally con-

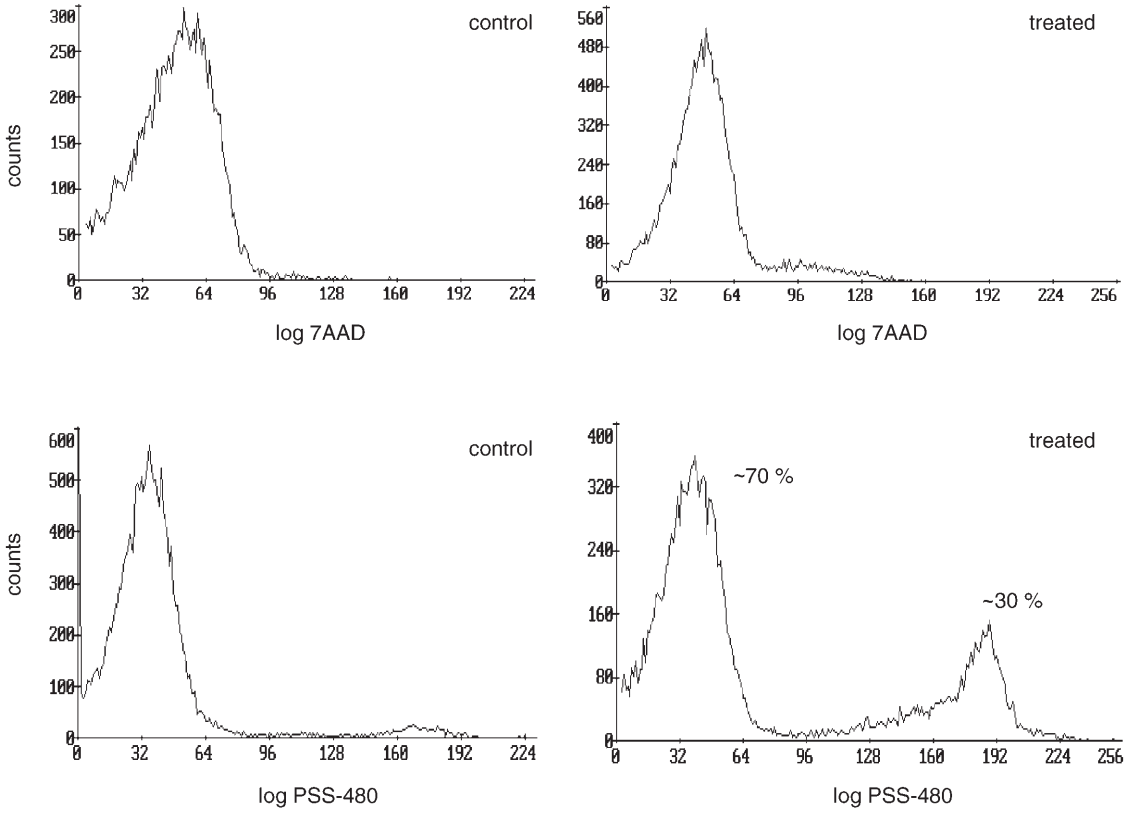


Figure 6. Flow-cytometry graphs illustrating staining of Jurkat cells by PSS-480 and 7AAD. Both control and treated cells exhibit similar levels of staining by 7AAD; this indicates that there is the same level of necrotic cells in the population (less than 5% in each case). Cells treated with camptothecin exhibit significantly more staining by PSS-480 than do control cells. Approximately 30% of treated cells were identified as apoptotic by using PSS-480, while less than 5% of the untreated cells were stained with PSS-480.

fined to the inner monolayer becomes distributed between the inner and outer leaflets.[25] The ability of Zn^{2+}–DPA coordination complexes to selectively bind to negatively charged membranes appears to be an effective strategy for recognizing cells in the early-to-middle stages of apoptosis. The Zn^{2+}–DPA coordination complexes presented here circumvent some of the limiting features of annexin V. For example, binding of the PS-affinity group to apoptotic cell membranes is Ca^{2+}-independent, the binding is virtually instantaneous, and the fluorescein in PSS-480 is compatible with the argon lasers found in commonly used flow cytometers. Furthermore, the presence of up to 10% serum in the binding medium has no adverse effect on staining, and apoptotic cells were successfully stained by PSS-480, PSS-biotin, and PSS-green QD at temperatures from 4 to 37 °C with incubation periods as short as 30 s (see Supporting Information). Similar results could not be obtained when fixed cells were used, and a nearly homogeneous staining of the cytosol of Jurkat cells was observed when cells were stained with PSS-480 after ethanol fixation.

The versatile PSS-biotin allows PS detection by a wide range of commercially available streptavidin–fluorophore conjugates. For example, the quantum dot–streptavidin conjugates are well suited for fluorescence microscopy because of the high quantum yield common to quantum dots,[26] as well as the ability of the quantum dots to resist photobleaching. A simplification of the quantum-dot system was achieved by directly conjugating the PS-affinity group to micelle-encapsulated CdSe/CdS quantum dots to afford a PS-selective stain (PSS-green QD) that can withstand prolonged exposures without diminished fluorescence intensity. In addition, the quantum-dot system can exhibit enhanced binding due to multivalency effects. A potential drawback, however, is the greater number of washings that must be performed after staining cells with PSS-green QD. The extreme brightness of the quantum dots means that a very high fraction of the unbound material must be eliminated in order to achieve a suitably dark background.

The PS-affinity group used in all three probes apparently binds only to the apoptotic cell surface. The fact that only surface binding is observed indicates that no membrane permeabilization has taken place and signals that the cells have not yet progressed to the later stages of apoptosis. The exclusion of PSS-green QD from the cell interior is noteworthy considering that similarly designed systems have been reported to cross the membranes of other cell lines when incubated for longer periods.[27] Another salient point is the difference in photophysics between the coordination complexes described here and PSS-380, our previously reported first-generation sensor for apoptosis.[20] Association of PSS-380 to a PS-rich membrane leads to fluorescence enhancement, due to enhanced binding of Zn^{2+} to the DPA units in PSS-380, which decreases photoinduced electron transfer (PET) quenching.[28] In contrast, the PET-quenching pathway is not significant in PSS-480, and control studies with vesicles show that association of PSS-480 with PS-rich membranes does not alter its fluorescence intensity. Similarly, the fluorescence intensity of PSS-green QD is unchanged upon membrane binding. In other words, the fluorescent probes presented here act in the same way as annexin V–FITC; that is, they are PS-selective stains that require a washing step to remove the unbound material.

Conclusion

In summary, we have shown how Zn^{2+}–DPA coordination complexes can be developed into effective fluorescent probes for apoptosis. The two Zn^{2+}–DPA subunits selectively bind to membranes enriched in anionic PS. The versatility of the system is enhanced by attaching a biotin reporter element (PSS-biotin); this makes detection of apoptotic cells possible with a range of fluorescent streptavidin conjugates. The Zn^{2+} coordination complexes allow users to identify apoptotic cells under Ca^{2+}-free conditions and with fast binding kinetics; this broadens the scope of the PS-detection method for apoptosis. Indeed, the low-molecular-weight, nonprotein probes presented might be adaptable to other imaging techniques, such as radiography and magnetic resonance spectroscopy.[29]

Experimental Section

Synthesis

Preparation of PSS-480-diacetate: 5-Carboxyfluorescein diacetate (0.097 g, 0.21 mmol) was added to a solution of *N*-hydroxysuccinimide (0.37 g, 0.32 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride salt (EDC; 0.61 g, 0.32 mmol) in dry CH_2Cl_2 under Ar. The reaction mixture was stirred for 1 h. Amine **1** (0.14 g, 0.21 mmol)[21] in dry CH_2Cl_2 was added. The reaction mixture was stirred overnight, then washed with water and brine and dried over Na_2SO_4. The solvent was removed under vacuum, and the residue was separated by chromatography on a silica column with $CHCl_3$/MeOH (92:8) as the eluent. Yield: 31%; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.31$ (s, 6H), 3.62 (br, 4H), 3.72 (m, 6H), 3.78 (m, 10H), 3.91(m, 2H), 4.18 (m, 2H), 6.71–6.80 (m, 4H), 6.88 (s, 2H), 7.04 (s, 1H), 7.09–7.14 (m, 5H), 7.19 (m, 2H), 7.30 (s, 1H), 7.54–7.64 (m, 8H), 8.20 (m, 1H), 8.43 (br, 1H), 8.48–8.50 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 21.3$, 40.3, 58.7, 60.1, 67.6, 69.9, 70.0, 70.6, 71.0, 110.7, 113.9, 116.0, 118.1, 122.1, 122.2, 122.4, 123.0, 123.3, 123.7, 124.7, 126.7, 129.0, 135.0, 136.7, 137.2, 140.7, 148.9, 149.1, 151.7, 152.4, 155.2, 159.0, 159.7, 165.7, 168.4, 169.0; FAB MS *m/z* 1090 $[M+H]^+$.

Preparation of PSS-480: PSS-480 diacetate (0.03 g, 0.028 mmol) was dissolved in MeOH (0.5 mL) and treated with NH_4OH (0.5 mL). The reaction mixture was stirred at 40 °C overnight. The solvents were then removed under vacuum, the residue washed with water (4×), and dried under high vacuum to yield 0.018 g (60%) of diammonium salt. The removal of acetoxy groups was confirmed by ^{1}H NMR. The Zn^{2+} complex was prepared by mixing a solution of diammonium salt (0.018 g, 0.17 mmol) in methanol and an aqueous solution of zinc nitrate (0.01 g, 0.35 mmol). After 30 min of stirring, the solvents were removed under vacuum, and the residue lyophilized to yield the zinc complex in quantitative yield.

Preparation of PSS-biotin: 1-Hydroxy 1*H*-benzotriazole (0.14 g, 1 mmol) and EDC (0.19 g, 1 mmol) were added to a solution of biotin (0.24 g, 1 mmol) in dry DMF under an Ar atmosphere. Amine **1** (0.65 g, 1 mmol) in dry CH_2Cl_2 was added, and the reaction mixture was stirred for 2 d. The solvent was then removed under vacuum, and the reaction mixture was taken up in $CHCl_3$. The $CHCl_3$ layer was washed with sat. $NaHCO_3$, water, and brine and dried over anhydrous $MgSO_4$. The solvent was removed under

vacuum, and the residue was separated by chromatography on a neutral alumina column with $CHCl_3$/MeOH (98:2) as the eluent. The overall yield of the uncomplexed compound was 60%. Selected data: ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 1.2$–1.64 (m, 6H), 2.03 (m, 2H), 2.55 (d, $J = 12.3$ Hz, 1H), 2.76–2.82 (q, $J = 5.1$ Hz, 1H), 3.05–3.09 (m, 1H), 3.39 (m, 2H), 3.51–3.53 (m, 2H), 3.57 (m, 6H), 3.70–3.74 (m, 10H), 4.04–4.14 (m, 5H), 4.26–4.30 (m, 1H), 6.36 (s, 1H), 6.42 (s, 1H), 6.83 (s, 2H), 7.08 (s, 1H), 7.22–7.26 (m, 4H), 7.57 (d, $J =$ 7.8 Hz, 4H), 7.70–7.76 (m, 4H), 7.83 (t, $J = 5.4$ Hz, 1H), 8.47–8.49 (m, 4H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 25.3$, 28.0, 28.2, 35.1, 38.4, 48.6, 55.4, 57.4, 59.2, 61.0, 66.9, 68.9, 69.2, 69.6, 69.8, 113.2, 121.0, 122.2, 122.4, 136.6, 140.2, 148.8, 158.4, 159.2, 162.7, 172.1; FAB MS m/z 874 $[M+H]^+$.

The uncomplexed compound (0.87 g , 1 mmol) in methanol and zinc nitrate (0.61 g, 2.05 mmol) in water were mixed, and this solution was stirred for 0.5 h. The solvents were removed under vacuum, and the resulting PSS-biotin was used without further purification.

Preparation of carboxy-PEG encapsulated quantum dots: Octadecylamine-stabilized CdSe/CdS core/shell nanocrystals (QDs; typical UV absorption $\lambda_{max} \sim 550$ nm) were prepared by following existing literature methods and redissolved in chloroform to provide a stock solution of 150 mg mL^{-1}.[22,23] The organic soluble QDs were then encapsulated in carboxy-PEG-phospholipid micelles[24] to render them water soluble. More specifically, an aliquot (300 μL) of the QD stock solution (150 mg mL^{-1}) was combined with a solution (~1 mL) of chloroform containing 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy poly(ethylene glycol)2000] ammonium salt (0.0165 g, 5.8×10^{-6} mol). After complete evaporation of the chloroform (by careful heating with stirring), the residue was further warmed to ~80 °C in a water bath for 1–2 min, after which time double deionized water (1 mL) was added with vigorous stirring. The sample was stirred for 1–2 min at ~80 °C and then sonicated at RT for 10 min to give an optically transparent solution. The sample was then centrifuged at 5000 rpm for 10 min to pellet out any unencapsulated or aggregated particles. The supernatant was transferred to a new vial and centrifuged at 500 000 *g* for 2 h to separate/pellet out the phospholipid-encapsulated QDs from the empty micelles remaining in the supernatant. The supernatant was carefully removed, and the QD–micelle pellet was resuspended in phosphate buffer (500 μL, pH 7.4).

The supernatant containing the excess phospholipid micelles was frozen with liquid nitrogen and lyophilized to determine the approximate amount of recovered phospholipids. The amount of phospholipid incorporation in the QD micelles could then easily be determined. In general, ~33% of the phospholipids were found to be utilized in the encapsulation of the QDs, giving $\sim 1.9 \times 10^{-6}$ mol of total phospholipid per 500 μL of QD solution.

Preparation of PSS-green QD: EDC (7.6 mg, 4.0×10^{-5} mol), *N*-hydroxysulfosuccinimide (sulfo-NHS, 5.7 mg, 3.0×10^{-5} mol), and the preformed $Zn(NO_3)_2$ complex of amine **1** (1.22×10^{-5} mol) were dissolved in the carboxy-PEG micelle-encapsulated QD solution [500 μL in phosphate buffer (pH 7.4)]. The resulting solution was mixed with mild agitation for 2 h and then purified by dialysis (Spectra/POR®, 50 000 MWCO) against double deionized water for 24 h to provide the $(Zn^{2+})_2$-DPA-PEG micelle-encapsulated quantum dots. The aqueous QD solution was stored at −24 °C in the dark until needed. These solutions exhibited no flocculation and retained their PS binding capability for at least one month.

Transmission electron microscopy: TEM samples were prepared by allowing a small drop of a chloroform nanoparticle solution on a copper-coated TEM grid to evaporate by air. The TEM images were obtained from a Philips CM30 microscope at 300 kV.

Cell staining and fluorescence microscopy: Annexin V and 7AAD were obtained from BD Biosciences (San José, CA). Quantum dot–streptavidin conjugates were from Quantum Dot Corporation (Hayward, CA). The Marina Blue streptavidin conjugate was from Molecular Probes (Eugene, OR). Jurkat cells were grown to a density of approximately 1.0×10^6 mL^{-1} in RPMI 1640, 10% FCS at 37 °C, 5% CO_2. A 10 mL volume of cells was treated with camptothecin (10 μM final concentration) in growth medium for 3.5 h at 37 °C, 5% CO_2. Cells were spun down and resuspended in 1X annexin binding buffer (10 mM HEPES sodium salt, 2.5 mM $CaCl_2$, 140 mM NaCl, pH 7.4) for experiments in which annexin V was used, or in a buffer of *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES; 5 mM), NaCl (145 mM), pH 7.4 for experiments in which annexin V was not used. Aliquots (0.5 mL) of the treated cells, along with controls, were then treated with the indicated staining reagents at the indicated concentrations. Annexin V-FITC was used according to the manufacturer's protocol (BD Biosciences). All reagents were added simultaneously. The cell suspensions were mixed thoroughly by repeated inversion and then incubated 15 min at 37 °C, except where temperature effects were being evaluated. Cells were then centrifuged, resuspended, and washed twice in TES (5 mM), NaCl (145 mM), pH 7.4 buffer. At this point, 250 μL of the suspension was transferred to a 16-well chamber slide for microscopy. Fluorescence microscopy was performed immediately following cell staining on an Axiovert S100 TV microscope (Carl Zeiss) equipped with filter sets DAPI/Hoechst/AMCA, FITC/RSGFP/Bodipy/Fluo3/DiO, Cy3 (Chroma, Rockingham, VT). Pictures were taken on a black and white digital camera (Photometrics, Tucson, AZ) and colored afterwards by using Photoshop 6.0 software (Adobe).

Flow cytometry: Jurkat cells were cultured according to the same procedures described for fluorescence microscopy. A 10.0 mL volume of cells was treated with camptothecin (10 μM final concentration) in growth medium for 16.5 h at 37 °C, 5% CO_2. Cells were spun down and resuspended in 1X annexin binding buffer (10 mM HEPES sodium salt, 25 mM $CaCl_2$, 140 mM NaCl, pH 7.4) for experiments in which annexin V was used, or in a buffer of TES (5 mM), NaCl (145 mM), pH 7.4 for experiments in which annexin V was not used. Cell aliquots (1.0 mL) were stained with 7AAD (500 ng mL^{-1}) and either PSS-480 (5 μM) or annexin V-FITC (5 μL mL^{-1}; BD Biosciences commercial solution). All reagents were added simultaneously. The cell suspensions were mixed thoroughly by repeated inversion and then incubated 15 min at 37 °C, except where temperature effects were being evaluated. Cells were then centrifuged, resuspended, and washed twice in TES (5 mM), NaCl (145 mM), pH 7.4 buffer. Flow cytometry was performed immediately after staining on an Epics XL flow cytometer (Coulter, Miami, FL) with an argon laser. FITC was analyzed by using a 520 nm bandpass filter, and 7AAD was analyzed by using a 580 nm bandpass filter. Software color compensation was used and data analysis was performed by using Multiplus AV Software (Phoenix Flow Systems, Dan Diego, CA).

Acknowledgements

This work was supported by the National Institutes of Health, the Phillip Morris External Research Program, Department of Defense, and Sandia National Laboratories. Sandia is a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy's National

Nuclear Security Administration under contract DE-AC04-94AL8500. The authors are grateful to Dr. Timothy J. Boyle (SNL) and Robin M. Sewell (SNL) for technical support in the CdSe/CdS-particle preparation, to Dr. Thomas J. Headley (SNL) for technical support with TEM, and to Dr. Jun Liu (SNL) and Professor Holly Goodson (UND) for technical assistance and useful discussion. Researchers who wish to obtain a sample of PSS-480 or PSS-biotin should contact the corresponding author.

Keywords: annexin V · apoptosis · fluorescent probes · phosphatidylserine · quantum dots

[1] J. M. Boon, B. D. Smith, *Med. Res. Rev.* **2002**, *22*, 251 – 281.
[2] J. A. F. Op den Kamp, *Annu. Rev. Biochem.* **1979**, *48*, 47 – 71.
[3] C. Pelassy, J. P. Breittmayer, C. Aussel, *Biochem. Pharmacol.* **2000**, *59*, 855 – 863.
[4] T. Matsura, B. F. Serinkay, J. Jing, V. E. Kagan, *FEBS Lett.* **2002**, *524*, 25 – 30.
[5] R. A. Schlegel, P. Williamson, *Cell Death Differ.* **2001**, *8*, 551 – 563.
[6] D. A. Bird, K. L. Gillotte, S. Horkko, P. Friedman, E. A. Dennis, J. L. Witztum, D. Steinberg, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 6347 – 6352.
[7] P. Williamson, S. van den Eijnde, R. A. Schlegel, *Methods Cell Biol.* **2001**, *66*, 339 – 364.
[8] M. Van Engeland, L. J. W. Nieland, F. C. S. Ramaekers, B. Schutte, C. P. M. Reutelingsperger, *Cytometry* **1998**, *31*, 1 – 9.
[9] B. Plaiser, D. R. Lloyd, G. C. Paul, C. R. Thomas, M. Al-Rubeai, *J. Immunol. Methods* **1999**, *229*, 81 – 95.
[10] J. F. Tait, D. F. Gibson, C. Smith, *Anal. Biochem.* **2004**, *329*, 112 – 119.
[11] J. Y. Lee, S. Miraglia, X. W. Yan, E. Swartzman, S. Cornell-Kennon, J. Mellentin-Michelotti, C. Brusco, D. S. France, *J. Biomol. Screening* **2003**, *8*, 81 – 88.
[12] J. A. Barnes, A. V. Gomes, *Mol. Cell. Biochem.* **2002**, *231*, 1 – 7.
[13] D. Arboledas, N. Olmo, M. A. Lizarbe, J. Turnay, *FEBS Lett.* **1997**, *416*, 217 – 220.
[14] J. Dachary-Prigent, J. M. Pasquet, J. M. Freyssinet, A. Nurden, *Biochemistry* **1995**, *34*, 11 625 – 11 634.
[15] D. Kamp, T. Sieberg, C. W. M. Haest, *Biochemistry* **2001**, *40*, 9438 – 9446.
[16] T. Lakko, L. King, P. Fraker, *J. Immunol. Methods* **2002**, *261*, 129 – 139.
[17] G. A. Wurth, A. Zweifach, *Biochem. J.* **2002**, *362*, 701 – 708.
[18] S. Bose, I. Tuunainen, M. Parry, O. P. Medina, G. Mancini, P. K. J. Kinnunen, *Anal. Biochem.* **2004**, *331*, 385 – 394.
[19] E. A. Schellenberger, F. Reynolds, R. Weissleder, L. Josephson, *ChemBioChem* **2004**, *5*, 275 – 279.
[20] A. V. Koulov, K. A. Stucker, C. Lakshmi, J. P. Robinson, B. D. Smith, *Cell Death Differ.* **2003**, *10*, 1357 – 1359.
[21] C. Lakshmi, R. G. Hanshaw, B. D. Smith, *Tetrahedron* **2004**, *60*, 11 307 – 11 315.
[22] S. D. Bunge, K. M. Krueger, T. J. Boyle, M. A. Rodriguez, T. J. Headley, V. L. Colvin, *J. Mater. Chem.* **2003**, *13*, 1705 – 1709.
[23] J. J. Li, Y. A. Wang, W. Guo, J. C. Keay, T. D. Mishima, M. B. Johnson, X. Peng, *J. Am. Chem. Soc.* **2003**, *125*, 12 567 – 12 575.
[24] B. Dubertret, P. Skourides, D. J. Norris, V. Noireaux, A. H. Brivanlou, A. Libchaber, *Science* **2002**, *298*, 1759 – 1762.
[25] S.-B. Ning, L. Wang, Z.-Y. Li, W.-W. Jin, Y.-C. Song, *Anal. Bot.* **2001**, *87*, 575 – 583.
[26] X. Michalet, F. F. Pinaud, L. A. Bentolila, J. M. Tsay, S. Doose, J. J. Li, G. Sundaresan, A. M. Wu, S. S. Gambhir, S. Weiss, *Science* **2005**, *307*, 538 – 544.
[27] A. M. Koch, F. Reynolds, H. P. Merkle, R. Weissleder, L. Josephson, *ChemBioChem* **2005**, *6*, 337 – 345.
[28] A. V. Koulov, R. G. Hanshaw, K. A. Stucker, C. Lakshmi, B. D. Smith, *Isr. J. Chem.* **2005**, *45*, 373 – 379.
[29] T. Z. Belhocine, J. F. Tait, J.-L. Vanderheyden, C. Li, F. G. Blankenberg, *J. Proteome Res.* **2004**, *3*, 345 – 349.

Received: April 7, 2005
Published online on November 7, 2005

