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Review

New reagents for phosphatidylserine recognition and detection of apoptosis

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Abstract—The phospholipid bilayer surrounding animal cells is made up of four principle phospholipid components, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM). These four phospholipids are distributed between the two monolayers of the membrane in an asymmetrical fashion, with PC and SM largely populating the extracellular leaflet and PE and PS restricted primarily to the inner leaflet. Breakdown in this transmembrane phospholipid asymmetry is a hallmark of the early to middle stages of apoptosis. The consequent appearance of PS on the extracellular membrane leaflet is commonly monitored using dye-labeled Annexin V, a 36 kDa, Ca^{2+}-dependent PS binding protein. Substitutes for Annexin V are described, including small molecules, nanoparticles, cationic liposomes, and other proteins that can recognize PS in a membrane surface. Particular attention is given to the use of these reagents for detecting apoptosis.

Contents

1. Introduction

The phospholipid bilayer surrounding animal cells is a dynamic environment made up of four principle phospholipid components,[1] phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM) (Fig. 1A). These four phospholipids are distributed between the two monolayers of the membrane in an asymmetrical fashion, with the choline-containing lipids, PC and SM, largely populating the extracellular leaflet, while the aminophospholipids, PE and especially PS, are restricted primarily to the inner membrane leaflet[2–6] (Fig. 1B). This membrane asymmetry has been known for some time, and there is a consensus that it is maintained by the concerted action of a family of translocase enzymes. Efforts to elucidate the structure and mechanism of these transport proteins are ongoing and are described elsewhere.[1,7,8] The primary objective of the present article is to summarize the recent attempts to develop sensing strategies that report a breakdown in this membrane asymmetry, a signature event indicating that cells have entered the early-to-middle stages of apoptosis.[9,10]

Apoptosis, or the sequence of cellular events collectively known as 'programmed cell death,' is an important

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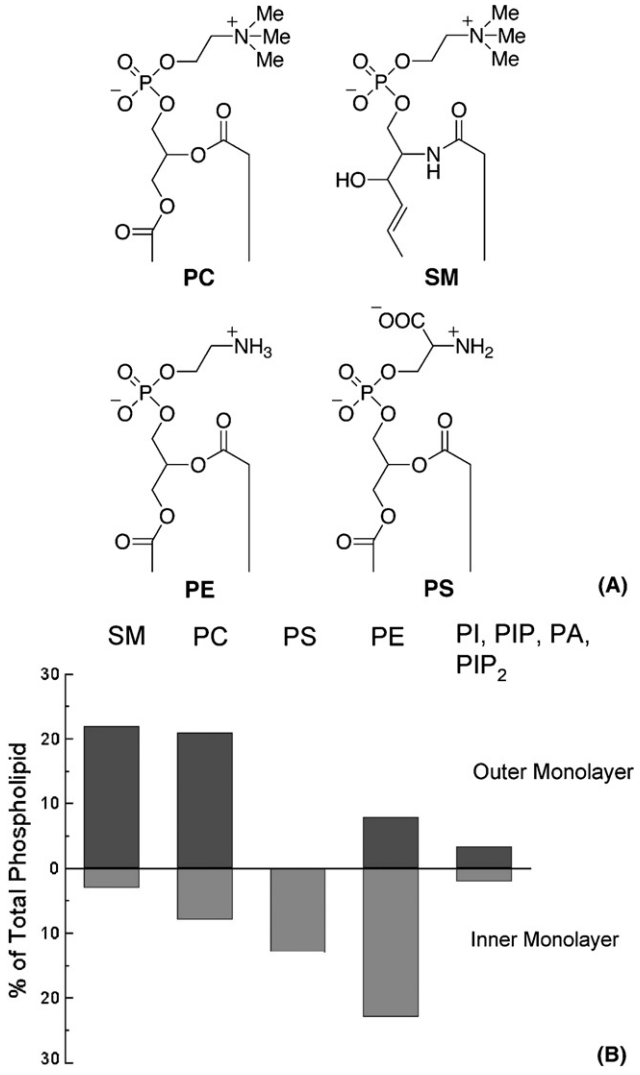


Figure 1. (A) Structure of the common animal cell membrane phospholipids phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylethanolamine (PE), and phosphatidylserine (PS). (B) Distribution of the common membrane phospholipids between the inner and outer monolayers of the membrane bilayer. Adapted from Ref. 2.

process whereby cells are intentionally marked for clearance from the body. Apoptosis is a central process in developmental biology and also in many types of diseases. For example, selective induction of apoptosis in cancerous tissue is an attractive chemotherapeutic strategy, and detection of apoptosis is therefore a key step in the drug development process. Various strategies for detecting apoptosis have been reported, including monitoring of intracellular caspase activity,[11] observing nucleic acid fragmentation,[12,13] and detection of membrane permeabilization.[14] These assays are employed as diagnostic tools for identifying apoptosis, but each has limitations that render it imperfect in certain situations.

Loss of the phospholipid asymmetry inherent to healthy animal cell membranes is a hallmark of apoptosis, regardless of the initiating stimulus.[13–15] During the early to middle stages of apoptosis, the PS normally found exclusively on the inner membrane monolayer becomes scrambled between the two membrane leaflets. PS is the only anionic phospholipid component of the plasma membrane, that is, present in appreciable levels, and externalization of PS results in a net buildup of anionic charge on the membrane surface.[16,17] There is abundant evidence suggesting that PS externalization is a contributing factor to the recognition of dead and dying cells by macrophages.[18–20] The externalized PS can be detected on the cell surface using indicator-labeled reagents that preferentially bind the PS headgroup.[21,22] PS externalization precedes the upregulation of protease activity in the cytosol, and occurs long before membrane permeabilization begins.[14] Another attractive feature of this cell surface assay is that it avoids the complications of other assays that require access to the cytosol. Furthermore, this strategy of PS recognition makes it possible to consider applications for site-specific in vivo imaging of apoptotic tissue that would be useful in the treatment of various diseases such as cancer and cardiovascular disease.

2. Annexin V as a reagent for detecting apoptosis

The annexins are a group of proteins with presently undetermined function, all of which bind anionic phospholipids in a Ca^{2+}-dependent manner.[23–26] One member of the family, Annexin V (Anx V), binds PS with high selectivity and high affinity,[21,23] making it well-suited for detection of apoptosis. A variety of fluorophore-labeled versions of Anx V are now commercially available, and detection of cell-surface PS by this technique has become a standard protocol in cell biology research.[21] The basic structural[27,28] and PS-binding[29–32] features of Anx V have been described in previous reports, and here we outline only the structural and biochemical features of Anx V that confer its PS-recognition capabilities, as well as highlight the limitations of Anx V that make development of non-protein PS recognition compounds a promising research topic.

A number of Anx V crystal structures have been solved,[33–41] and the Ca^{2+} coordination and PS binding geometry is well characterized. Anx V is a 36 kDa, single stranded protein. Its structure contains four domains, each consisting of five alpha-helical regions, and a short N-terminal sequence, making it unique among annexins, most of which have more elaborate N-terminal residues, which are thought to confer a specific functionality. While the challenge of achieving a crystal structure of a membrane-bound Anx V has yet to be overcome, a structure of Anx V bound to glycerophosphoserine has been reported.[42] In this structure, two Ca^{2+} ions are coordinated to the PS headgroup, one bound to the phosphoryl oxyanion and the other to the carboxylate (Fig. 2). Docking studies later revealed a PS binding consensus sequence of Arg^{25}-X_3-$Lys^{29}\cdots Arg^{63}$-X_4-Asp^{68}-X_2-Ser^{71}-Glu^{72}, which was subsequently shown to be present in either domain 1 or 2, or both, in all known annexins.[43,44] No other phospholipid has the same charge separation and geometrical orientation as

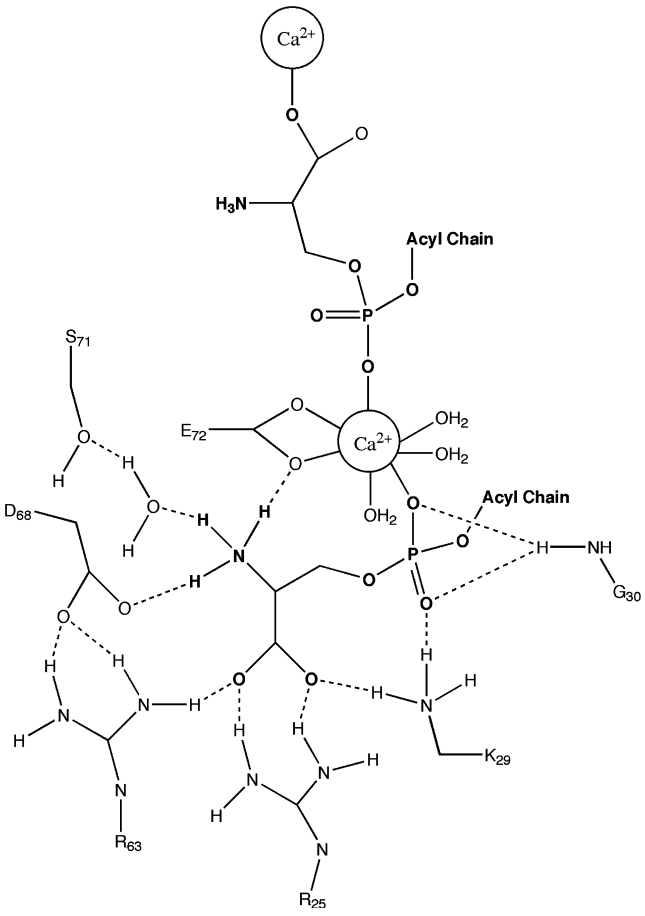


Figure 2. A PS-binding site in domain 1 of Anx V. Two bound PS molecules are shown coordinated to two Ca^{2+} ions. Detailed hydrogen bonding patterns are shown for those conserved residues that constitute the PS-binding consensus sequence. Adapted from Ref. 48.

PS, which suggests that the Anx V protein has evolved specifically to perform PS-binding functions.

As previously mentioned, appearance of PS on a cell surface is a general indicator of apoptosis, and binding of dye-labeled Anx V to cell membranes enriched in PS has become a widely used technique for detecting apoptosis using flow cytometry[21,45] and fluorescence microscopy.[22] A number of Anx V conjugates bearing various reporter elements have been prepared and used for detection of apoptosis, and methodology for preparing customized Anx V conjugates has been described.[46] Josephson and co-workers have reported that Anx V retains its PS binding capabilities as long as the extent of modification does not exceed 1.6 modifications per mole of protein. The applications for which Anx V conjugates have been prepared are diverse, and include: FITC-labeled[21,45] and Phycoerythrin-labeled[47,48] Anx V for flow cytometry and fluorescence-activated cell sorting (FACS), Cy5.5 conjugated Anx V for near-infrared (NIR) optical imaging,[49–51] Anx V-^{18}F conjugates for positron emission tomography (PET),[52–54] Anx V-Tc-99m conjugates for radioimaging,[55–57] biotinylated Anx V with labeled streptavidin conjugates for a variety of applications,[58–60] and metal and nanoparticle Anx V conjugates for cell separation and magnetic resonance imaging.[61,62]

Even though Anx V derivatives are widely used for PS-sensing applications and apoptosis detection, Anx V is not without limitations. For instance, the unfunctionalized protein has a mass of approximately 36 kDa, which restricts its use to those applications where a PS sensor of this size can be accommodated. Furthermore, Anx V-PS binding requires millimolar levels of Ca^{2+} in order to produce the nanomolar dissociation constants that make using the protein desirable.[24–26] This level of Ca^{2+} may be problematic in situations where other processes are to be monitored simultaneously. Additionally, animal cells frequently have integral membrane phospholipid transport proteins, called 'scramblases,' that can move phospholipids nonspecifically between the two membrane monolayers. These scramblases are activated by micromolar Ca^{2+} levels,[8] well below that necessary for Anx V-PS binding. This raises the possibility that false positives may occur when using Anx V to detect apoptosis. The rate of Anx V-PS binding is also quite slow. Complete membrane binding by Anx V often requires incubation periods of up to 1 h,[63] which is problematic for many types of kinetic assays. The biochemical stability of Anx V has been discussed in previous reports, particularly noting that Anx V is susceptible to N-terminal proteolytic degradation.[64,65] The aggregate of these limitations suggests that alternative, non-protein, sensors for PS would have multifarious applications.

3. Small molecule annexin mimics

Alternative sensing strategies for apoptotic cell membranes have included small molecules, surface-functionalized nanoparticles, and supramolecular assemblies. The idea that small molecules are able to signal changes in cell membrane properties is supported by use of the fluorescent dyes MC 540[66] and FM 1-43FX[67] (Fig. 3),

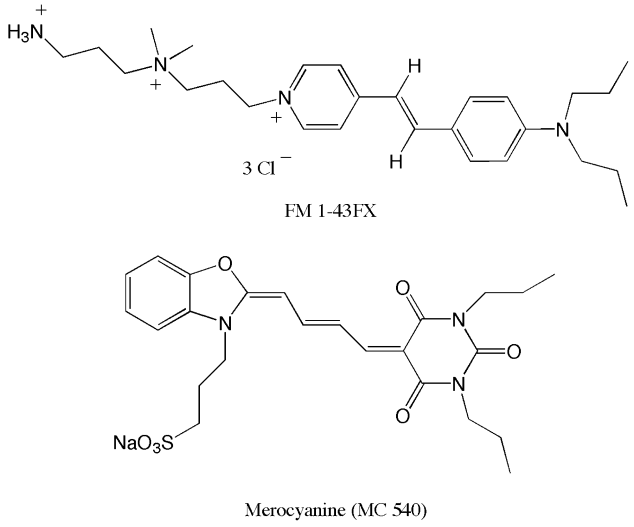
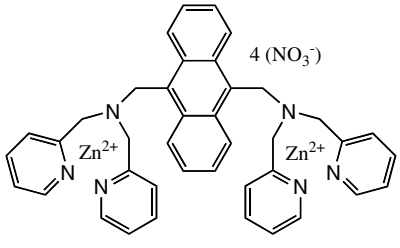


Figure 3. Structure of the membrane dyes FM 1-43FX and Merocyanine (MC 540) used to detect apoptosis.

which have been evaluated for apoptosis detection. The binding of MC 540 to membranes is sensitive to membrane composition, and binding increases when cells become apoptotic.[9,68] However, there are significant disadvantages with MC 540. The signal difference between normal cells and apoptotic cells is only about 5-fold (it is up to 100-fold with Annexin V-FITC); furthermore, the dye is phototoxic. Likewise, FM 1-43FX is a cationic dye that binds more tightly to membranes that are enriched in anionic phospholipids than those of normal healthy cells, but gives a signal difference between normal and apoptotic cells of only 6–10-fold. Another disadvantage of FM 1-43FX is its broad emission (515–595 nm), which makes it difficult to simultaneously use a second fluorophore that emits in a range similar to that of fluorescein or rhodamine, two common emission channels in flow cytometry and fluorescence microscopy.

An effective small molecule mimic of Anx V should identify apoptotic cells with a large signal difference, but should not itself be toxic. Our group has demonstrated the ability of Zn^{2+}-2,2′-dipicolylamine (Zn^{2+}–DPA) coordination compounds to detect apoptotic cells.[69] The Zn^{2+}–DPA complex shown below, which we call PSS-380, was originally reported by the Himachi group as a fluorescent sensor for phosphorylated peptides.[70] The simultaneous coordination of two divalent metal cations by the organic scaffolding of PSS-380 forms a complex, that is, functionally similar to the Ca^{2+}-binding site of a single domain of Anx V,[42] the portion responsible for membrane binding and PS recognition. The spatial separation of the two Zn^{2+} ions enables PSS-380 to interact with both the carboxylate and phosphate anions present in the PS headgroup. The Ca^{2+} binding sites in Anx V differ in their affinity for Ca^{2+} in the same way as the two Zn^{2+} binding sites in the organic scaffold of PSS-380 differ in their affinity for Zn^{2+}. The first Zn^{2+} ion is bound with a K_a of approximately $10^7\ M^{-1}$, while the second Zn^{2+} is bound with an affinity of only around $10^4\ M^{-1}$.[71] This difference means that PSS-380 exists in solution predominately as the mono-Zn^{2+} form, and only after association of the first Zn^{2+}–DPA arm of the sensor with the anionic membrane surface does the second Zn^{2+} binding event take place. The binding of this second Zn^{2+} to the DPA ligand eliminates PET quenching by the DPA tertiary amine, and thus a fluorescence enhancement is observed when PSS-380 associates with an anionic membrane surface. This 'three component assembly process', consisting of the membrane surface, metal cation(s), and PS-binding group, mimics the recognition mechanism of Anx V (Fig. 4).



PSS-380

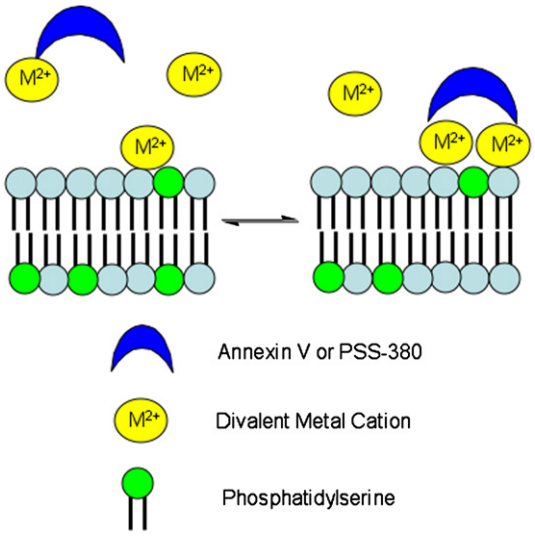


Figure 4. The three-component assembly process that results in high affinity association of Annexin V or PSS-380 with PS-rich membranes. The divalent cations, Ca^{2+} in the case of Annexin V, or Zn^{2+} in the case of PSS-380, induce strong association of the organic scaffold with the anionic PS headgroups.

Apoptotic cells can be effectively identified after staining with PSS-380, and independent applications of PSS-380 in cell biology research have been reported by other groups,[72,73] but there are limitations with its use. The anthracene fluorophore, which also serves as the sensor scaffold in this case, requires excitation at 380 nm, which falls outside the normal operational range of common flow cytometers. Further, PSS-380 suffers from rapid photobleaching, making it challenging to acquire time-dependent images. For these reasons we developed second generation Zn^{2+}–DPA compounds with different fluorophores.

A PS binding group, with two *meta*-oriented Zn^{2+}–DPA units,[74] was conjugated to either fluorescein, in the case of PSS-480, or to biotin in the case of PSS-Biotin (Fig. 5). The latter can be visualized by treatment with an appropriately labeled streptavidin conjugate.[75] Apoptotic Jurkat cells were stained with both PSS-480 and PSS-Biotin and detected readily by fluorescence microscopy. Binding of PSS-480 and PSS-Biotin was virtually instantaneous over the temperature range of 4–37 °C, and staining could be accomplished directly in cell culture media containing up to 10% serum. Binding of these compounds to cell membranes in the absence of Ca^{2+} is one advantage over Anx V. The fluorescein fluorophore of PSS-480 makes it particularly suitable for statistical analysis by flow cytometry, and similar levels of apoptosis were detected in a Jurkat cell population using both PSS-480 and Anx V-FITC. We also developed a quantum dot system for detecting PS on a cell surface.[75] By coating the surface of a CdS/CdSe quantum dot with phospholipids,[76] a water-soluble assembly was created, which allowed conjugation of Zn^{2+}–DPA

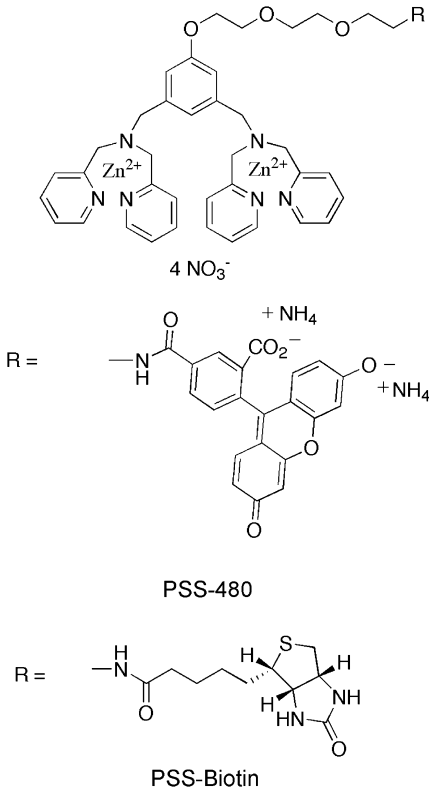


Figure 5. Structures of PSS-480 and PSS-Biotin.

as the PS-binding group. This stable supramolecular assembly allowed us to detect apoptosis with the same specificity as PSS-480 and PSS-Biotin but with the enhanced fluorescence properties of a quantum dot.

Zn^{2+}–DPA complexes have also been employed in indicator displacement assays that detect membranes enriched with PS.[77] In these assays, the Zn^{2+}–DPA systems shown in Figure 6 were used to form reversible complexes with either the UV–vis dye Pyrocatechol Violet or a fluorescent Coumarin Sulfonate. In the presence of liposomal membranes enriched in PS, the anionic PS headgroup displaced the indicator from the indicator–receptor complex, resulting in a change in the indicator absorbance or fluorescence emission intensity. These PS sensing systems did not respond to monodisperse short-chain PS, and also failed to respond to phospholipid vesicles containing the anionic phospholipids PA or PG. Thus, the binding of the Zn^{2+}–DPA complexes to PS-rich membranes requires a unique combination of both the anionic PS headgroup and a membrane surface.

4. Other sensing strategies

Other groups have used functionalized quantum dots and nanoparticles to monitor membrane associated biological processes,[78] including detection of apoptosis.[79] Schellenberger et al. generated a library of aminated dextran caged iron oxide nanoparticles, which were subsequently functionalized with cationic peptide sequences by disulfide bond formation between the nanoparticle surface and a side chain cysteine residue. In the best case, a 11-fold increase in fluorescence intensity was observed between normal and apoptotic cells when treated with nanoparticles functionalized with the cationic sequence RRRGRRRGC-SH. Though electrostatic interactions undoubtedly play a dominant role in the binding of these cationic nanoparticles to the apoptotic cell surface, no specific cell surface binding target was identified.

Additional strategies for detecting apoptosis based on changes in the plasma membrane have involved binding to apoptotic cells by the supramolecular assembly of cationic dye-labeled liposomes that bind anionic phospholipids. Bose et al. constructed cationic liposomes composed of POPC and containing the gemini surfactant *SS*-1 ((2*S*,3*S*)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)butane dibromide) and the fluorescent lipid analog DOPRho (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B solfonyl)).[80] These cationic liposomes were found to homogeneously stain the cell membranes of apoptotic cells, with only slight and highly irregular binding to healthy cells. Binding of these cationic dye-labeled liposomes appears to be mediated through electrostatic interactions between the cationic gemini surfactant and the anionic PS exposed on the cell surface.

Lastly, proteins other than Anx V have been used as sensors for apoptosis when conjugated to an appropriate fluorophore, contrast agent, or other reporter element. The C2A domain of the protein synaptotagmin I has been shown to bind anionic phospholipids in a Ca^{2+}-dependent manner, and Jung et al. have capitalized on this property of the C2A domain by engineering a biotinylated version of the protein, which was subsequently used as an MRI contrast agent following treatment with a Gd^{3+}-chelated streptavidin conjugate.[81] In a similar application, Heyder et al. designed a FITC-labeled lectin conjugate from *Narcissus pseudonarcissus* that could be used to detect apoptosis by exploiting the cell surface exposure of modified carbohydrate species that appear during early apoptosis.[82] While both reporter-conjugated proteins could be used effectively in assays for apoptosis, they retain many of the same limitations of Anx V.

5. Summary

Dye-labeled Anx V is currently a very popular protein-based reagent for detecting the PS that appears on the surface of apoptotic cells. However, advances in molecular recognition and nanoparticle fabrication are rapidly making available a series of alternative sensing systems. These improved reagents will present new opportunities to advance our understanding of the process of cell death. More effective strategies for identifying apoptosis will also open the door to applications such as in vivo imaging of apoptotic tissue, chemical regulation of cell cycle progression, and perhaps eventually the development of individualized treatment strategies for various types of illness.

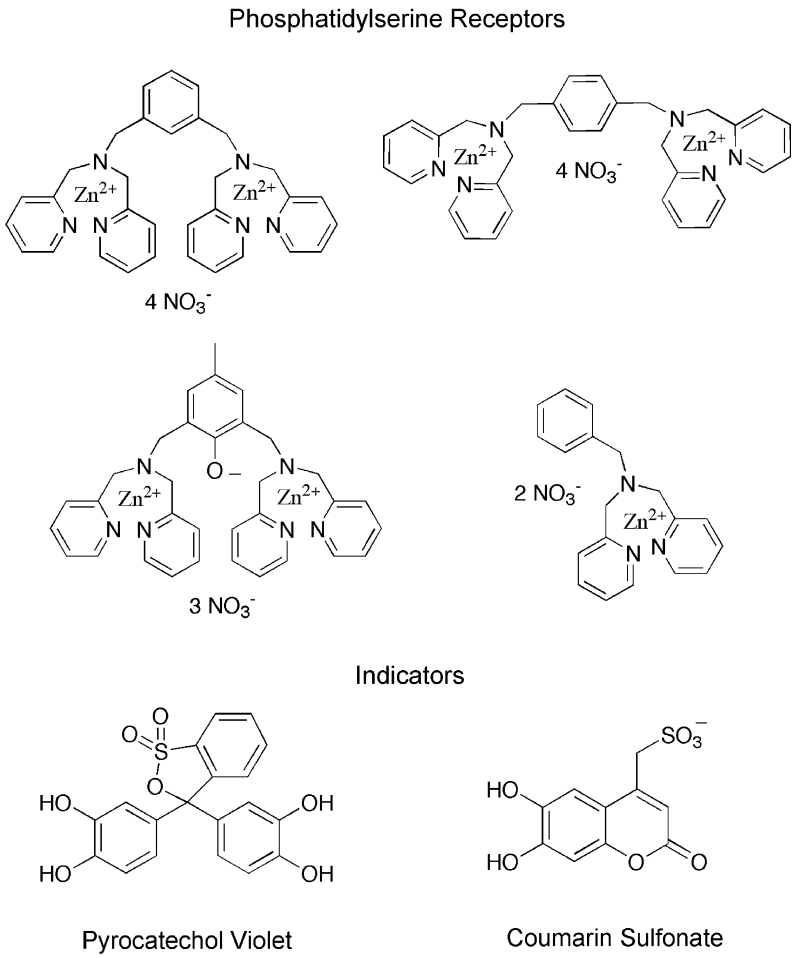


Figure 6. Structure of the Zn^{2+}–DPA coordination complexes used as PS receptors and the indicators used to form the reversible assemblies employed in the displacement assay for PS-rich liposomal membranes.

Acknowledgments

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