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Intraoperative Detection of Cell Injury and Cell Death with an 800 nm Near-Infrared Fluorescent Annexin V Derivative

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Abstract

The intraoperative detection of cell injury and cell death is fundamental to human surgeries such as organ transplantation and resection. Because of low autofluorescence background and relatively high tissue penetration, invisible light in the 800 nm region provides sensitive detection of disease pathology without changing the appearance of the surgical field. In order to provide surgeons with real-time intraoperative detection of cell injury and death after ischemia/reperfusion (I/R), we have developed a bioactive derivative of human annexin V (annexin800), which fluoresces at 800 nm. Total fluorescence yield, as a function of bioactivity, was optimized *in vitro*, and final performance was assessed *in vivo*. In liver, intestine and heart animal models of I/R, an optimal signal to background ratio was obtained 30 min after intravenous injection of annexin800, and histology confirmed concordance between planar reflectance images and actual deep tissue injury. In summary, annexin800 permits sensitive, real-time detection of cell injury and cell death after I/R in the intraoperative setting, and can be used during a variety of surgeries for rapid assessment of tissue and organ status.

Keywords

Annexin V; Near-Infrared Fluorescence; Intraoperative Imaging; Cell Injury; Cell Death; Acute Rejection; Ischemia/Reperfusion

INTRODUCTION

Cell injury and cell death caused by ischemia/reperfusion (I/R) are detrimental in many clinical situations such as organ transplantation and resection of the heart (1), lung (2), liver (3),

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pancreas (4), intestine (5) and kidney (6). Although intraoperative evaluation of tissue status is of critical importance to surgeons, there are few methods available for this purpose. Invasive biopsy requires long processing times, and is complicated by sampling error and bleeding (7, 8). pH probes provide real-time assessment of tissue acidity, but again, are limited to small area sampling (9,10). An ideal method for the assessment of tissue status would provide instantaneous evaluation of the entire surgical field.

Annexin V is a naturally-expressed, 36 kDa single chain protein that binds with nanomolar affinity ($K_d \approx 0.5\text{--}7\text{ nM}$) to phosphatidylserine (PS) in a calcium-dependent fashion (11,12). PS, normally sequestered on the inner leaflet of the lipid bilayer, is exposed at high concentrations on the cell surface when cell injury occurs, or during either apoptosis or necrosis. This externalization of PS occurs early in the cell death process, well before DNA fragmentation and nuclear condensation. Annexin V is widely used as one of the marker proteins for cell injury and cell death *in vitro* (13,14).

Recently, human studies utilizing radiolabeled annexin V have confirmed a pattern of biodistribution and pharmacokinetics suitable for *in vivo* evaluation of cell injury and cell death after acute cardiac transplant rejection, acute myocardial infarction, chemotherapy and radiotherapy (12). Radiolabeled annexin V, however, requires long integration times (typically 15–30 min) for imaging, and is associated with exposure of patients and caregivers to ionizing radiation. Other derivatives of annexin V used for imaging include a superparamagnetic nanoparticle that generates contrast for magnetic resonance imaging (MRI) (15), a Cy5.5 visible (far-red) fluorescent derivative for optical imaging (16), and a combination of the two (17). However, MRI is currently not a viable imaging technique for the operating room, and visible fluorescence suffers from high autofluorescent background, scatter, limited penetration, and absorption (reviewed in (18)).

Near-infrared (NIR) wavelengths, especially those in the 800 nm region, provide relatively high tissue photon penetration and low autofluorescent background (reviewed in (19)). Most importantly, heptamethine indocyanine fluorophores with excellent quantum yields are also available in this wavelength range, and can be used to create application-specific contrast agents for intraoperative imaging. We have previously developed an intraoperative NIR fluorescent imaging system, which in conjunction with an NIR fluorescent contrast agent, allows the surgeon to assess anatomy (with color video) and function (with NIR fluorescence) simultaneously and in real-time with ultra-high resolution (20,21). To date, this system has been applied to sentinel lymph node mapping (22), tissue calcification assessment (23) and the delivery of gene therapy (24). In this study, we show how it can be used to monitor tissue status during ischemia/reperfusion injury.

MATERIALS AND METHODS

Reagents

The N-hydroxysuccinimide (NHS) ester (CW800-NHS) and carboxylic acid (CW800-CA) forms of IRDyeTM 800CW NIR dye were provided as dry powders from LI-COR (Lincoln, NE). They were resuspended at 30 mM in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO) under reduced light conditions and stored at -80°C . Etoposide was purchased from Sigma. Annexin V, 10 mg/ml in phosphate-buffered saline (PBS), pH 7.4, was from Theseus Imaging Corporation (Boston, MA) and was stored at 4°C . Terminal dUTP nick end labeling (TUNEL) assay was performed using a Dead EndTM Fluorometric TUNEL System (Progenia, Madison, WI).

Conjugation Reactions

All steps were performed under reduced light conditions. Reactions contained 5 mg/ml annexin V and various molar ratios of CW800-NHS in phosphate buffered saline (6 mM phosphate, 150 mM NaCl, pH 7.8; PBS) with total reaction volumes ranging from 100 μ L (analytical) to 5 ml (preparative). Conjugation was initiated by adding CW800-NHS, and constant agitation (without frothing) was continued for 2 hrs at RT. Quenching of unreactive NHS esters was not necessary given the purification system used (see below).

Gel-Filtration Chromatography

The gel-filtration chromatography system consisted of an ÄKTA prime pump with fraction collector (Amersham Biosciences, Piscataway, NJ) and Econo-Pac P6 chromatographic cartridge with a cut-off of 6,000 Da (Bio-Rad, Hercules, CA). Gel-filtration, and on-line absorbance and fluorescence spectrometry was performed as described in detail previously (25). After conjugation, the sample was loaded into the injector and run at a flow rate of 1 ml/min using PBS, pH 7.8 as mobile phase. Full spectrum absorbance and fluorescence data were recorded every 10 sec. Desired products were collected by the fraction collector, pooled, and stored at 4°C in the dark without preservatives until use. Average yield was 80% or greater. The labeling ratio and the concentration of the conjugated protein (annexin800) were estimated using the extinction coefficients of annexin V ($\epsilon_{280\text{nm}} = 38,800 \text{ M}^{-1}\text{cm}^{-1}$) and CW800-CA ($\epsilon_{777\text{nm}} = 289,000 \text{ M}^{-1}\text{cm}^{-1}$) in 20% methanol in PBS, with correction for the 6.5% of measured absorbance at 280 nm due to CW800-CA:

$$\text{Labeling Ratio} = (\text{Abs}_{777\text{nm}} / \epsilon_{777\text{nm}}) / ((\text{Abs}_{280\text{nm}} - 0.065 * \text{Abs}_{777\text{nm}}) / \epsilon_{280\text{nm}})$$

MALDI-TOF Mass Spectrometry

Molecular weights of intact annexin V and annexin800 were measured using MALDI-TOF mass spectrometry on a Voyager-DE (Applied Biosystems, Foster City, CA) as previously described (25). 1 μ g of protein in PBS, pH 7.8 was desalted with a ZipTip_{C4} pipette tip (Millipore, Billerica, MA), equilibrated with 100% acetonitrile, 50% acetonitrile with 0.05% trifluoroacetic acid (TFA), and 0.1% TFA sequentially. Samples were loaded with the matrix 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), and the mass spectrum was obtained from 100 shots using positive reflector mode and a laser intensity of 2600–2800V, with a spectral range of 30,000–50,000 Da. Data were analyzed with Data Explorer (Applied Biosystems) software.

ES-TOF Mass Spectroscopic Site Mapping

230 μ L of 8 M urea/400 mM ammonium bicarbonate solution was added to 500 μ g annexin V or annexin800 in 20 μ L of PBS, pH 7.8. 5 μ L of 45 mM dithiothreitol (DTT) was added and incubated for 15 min at 50°C. 5 μ L of 100 mM iodoacetamide was added and incubated for 15 min at RT. The mixture was diluted with 750 μ L of buffer (80 mg ammonium bicarbonate and 6 mg CaCl_2 in 10 ml H_2O), 20 μ g of TPCK-trypsin (L-1-tosylamide-2-phenylethyl ketone-treated, Sigma) in H_2O was added, and the solution was incubated for 24 hr at 37°C. 20 μ L of this peptide digest was used for analysis on a Waters (Milford, MA) LCT ES-TOF LC/MS equipped with dual wavelength absorbance detector (Waters), multi-wavelength fluorescence detector (Waters), a Sedex Model 75 evaporative light scatter detector (ELSD; Richards Scientific, Novato, CA), and a lock-spray. The absorbance detector was set to 254 and 700 nm (the maximum permitted wavelength), and fluorescence detector was set to excite at 770 nm and detect emission at 800 nm. Leucine enkephalin (0.5 ng/ μ L) was used as a mass reference. Buffer A was 10 mM triethylammonium acetate, pH 7 (Glen Research, Sterling, VA) and buffer B was acetonitrile. Peptides were resolved on a 2.1 $\times$ 150 mm Symmetry C₁₈ column (Waters) at a flow rate of 0.3 ml/min, using a gradient of 5% to 40% B over 35 min. Mass was measured in ES+ mode. Data were analyzed with MassLynx (Waters) software, and expected peptide

masses were calculated from the mass obtained from Peptidemass (<http://au.expasy.org/tools/peptide-mass.html>) with the addition of the mass of CW800-CA (1003.24) and subtraction of the mass of H₂O (18.02). 3-D protein structure was visualized on a Macintosh iMac computer running RasMac Molecular Graphics version 2.6-ucb1.0 (University of California, Berkeley, CA).

Biopotency of Annexin800

Biopotency of fresh (prepared the same day) and stored (4°C in the dark for 3 months) annexin800 (labeling ratio = 1.2) was measured using a Biacore® 2000 (Biacore AB, Uppsala, Sweden; (26)), equipped with a L1 sensor chip coated with PS, or phosphatidylcholine (PC) as a control (27). The samples were mixed in 50 mM HEPES, pH 7.4 and 100 mM NaCl containing varying calcium concentrations. The equilibrium value of specific annexin-membrane binding at each calcium concentration was measured as the change in resonance units (RUs) seen on the PS chip subtracting the change observed on the PC chip. Relative RU was calculated with the maximum RU as 1.0. EC₅₀ was determined by fitting the titration data to a model of equilibrium binding to homogenous sites.

Intraoperative Imaging System

The intraoperative NIR fluorescence imaging system optimized for large animal surgery has been described in detail previously (20). Briefly, it is composed of two wavelength-isolated excitation sources, one generating 0.5 mW/cm² 400–700 nm “white” light, and the other generating 5 mW/cm² 725–775 nm light over a 15 cm diameter field of view. Simultaneous photon collection of color video and NIR fluorescence images is achieved with custom-designed optics that maintains separation of the white light and NIR fluorescence (>795 nm) channels. Spatial resolution at a field-of-view of 20 × 15 cm is 625 μm, and at a field-of-view of 4 × 3 cm is 125 μm. After computer-controlled (LabVIEW) camera acquisition via custom LabVIEW (National Instruments, Austin, TX) software, anatomic (white light) and functional (NIR fluorescence light) images can be displayed separately and merged. To create a single image that displays both anatomy (color video) and function (NIR fluorescence), the NIR fluorescence image was pseudo-colored in lime green and overlaid with 100% transparency on top of the color video image of the same surgical field. All images are refreshed up to 15 times per second. The entire apparatus is suspended on an articulated arm over the surgical field, thus permitting non-invasive and non-intrusive imaging.

In Vitro Cell Injury Detection

U937 leukemic cells were treated with or without 50 μM etoposide for 6 hrs in RPMI supplemented with 10% FBS. Cells were washed and resuspended in Hank's balanced salt solution (HBSS) containing 50 nM (dye concentration) of annexin800 (labeling ratio = 1.2 or 2.2), or CW800 conjugated to human serum albumin (HSA800; labeling ratio = 3.0; (25)) as a control, and incubated at RT in the dark for 30 min. Cells were washed, cytospun onto glass slides and photographed for phase contrast and NIR fluorescence using a Nikon Eclipse TE300 microscope as described previously (23). A total of 1,000 cells were quantified for each condition.

Intraoperative Cell Injury and Death Detection

Animals were housed in an AAALAC-certified facility and were studied under the supervision of an approved institutional protocol. 300–350 g Wistar male rats were purchased from Charles River Laboratories (Wilmington, MA), and 15–20 kg hound cross dogs were purchased from Marshall Bio Resources (North Rose, NY). All animals acclimated to the animal facility for at least 48 hrs prior to experimentation, and were euthanized after experimentation using

pentobarbital (rats) or rapid intravenous injection of Fatal-Plus (Vortech Pharmaceuticals, Dearborn, MI; dogs).

For I/R of rat liver, the right hepatic artery and corresponding portal vein were clamped with a surgical clip (Roboz, Gaithersburg, MD) for 1 hr, and reperused for 2 hrs. For I/R of rat intestine, a branch artery of the superior mesenteric artery and its corresponding vein, and both sides of the occupying intestine, were clamped for 1 hr with a surgical clip (Roboz) and mosquito clamp, respectively, and reperused for 2 hrs. After intravenous injection of 1.2 mg/kg annexin800 protein (labeling ratio = 1.2; 40 nmol/kg of fluorophore total), signal to background ratio (SBR) was measured every 1 min over the course of 60 min. The background region of interest was abdominal wall for liver I/R, and skin for intestinal I/R.

For I/R of the canine heart, a branch of the left anterior descending (LAD) artery was occluded with a tourniquet for 2 hrs and released for 2 hrs. After intravenous injection of 0.3 mg/kg annexin800 protein (labeling ratio = 1.2; 10 nmol/kg of fluorophore total), signal to background ratio (SBR) was measured every 1 min over the course of 60 min. After imaging, the heart was removed, sectioned at 1 cm intervals, and incubated with 1% 2,3,5-triphenyl tetrazolium chloride (TTC; (28)) at 37°C for 30 min in the dark.

Histopathology and Immunofluorescence Microscopy

After imaging, tissue sections from I/R and normal areas were placed in histology cassettes and embedded in Tissue-Tek® O.C.T. compound (Sakura Finetek USA, Torrance, CA), and frozen immediately in liquid nitrogen. Consecutive sections were stained by hematoxylin and eosin (H&E) or left unstained. TUNEL staining was performed on unstained sections by fixing in 1% paraformaldehyde in PBS for 15 min at RT, washing in PBS, washing in PBS supplemented with 1% Tween-20, and fixing again in 1% paraformaldehyde in PBS for 15 min at RT. Manufacturers instructions were then completed, followed by counterstaining of nuclei using 4',6-diamidino-2-phenylindole (DAPI), and mounting in Fluoromount-G (Southern Biotech, Birmingham, AL). For each microscope field, H&E, DAPI staining (blue fluorescence), TUNEL staining (green fluorescence), and annexin800 (NIR fluorescence) were visualized on a on a four-channel microscope described previously by our group (29).

RESULTS

Synthesis and Optical Properties of Annexin800

After reaction with fluorophore, annexin800 was successfully separated from free NHS ester and free CW800-CA, with a purity of >98% by gel-filtration (data not shown). Annexin800 absorbance showed a prominent peak at 700 nm in PBS that decreased in the presence of methanol, suggesting dye aggregation in PBS (Figure 1A; (30)). The absorbance and emission peaks of annexin800 were 777 nm and 797 nm, respectively (Figure 1A). As expected, the labeling ratio increased as the mixing ratio increased (Figure 1B). However, as the labeling ratio increased, the per-fluorophore fluorescence decreased and the per-molecule fluorescence peaked (Figure 1C). Due to this phenomenon, it was concluded that maximal per-molecule fluorescence yield occurs at a labeling ratio of 1.2 (Figure 1C). MALDI-TOF mass spectrometry confirmed that annexin800 is a mixture of annexin V substituted with various numbers of CW800, and validated the accuracy of the calculation of labeling ratio from the absorbance scan (Figure 1D).

Identification of Fluorophore Location(s) on Annexin800

In order to determine the conjugation site(s) of CW800, we performed tryptic mass-spec fingerprinting. The total ion chromatogram (TIC) demonstrated that peptides from both annexin V and annexin800 were well separated, with a small number of peaks of 700 nm

absorbance (the highest available wavelength on our detector) and 800 nm fluorescence were observed from annexin800, but not from annexin V (data not shown). By measuring the mass of the peptides with 700 nm absorbance and 800 nm fluorescence, we obtained two binding sites (Table 1). Based on the crystal structure (31), each substitution site is at the surface of the protein, and fluorophores are located relatively close to each other, but away from the calcium binding sites that are required for PS binding (Figure 2). One of the two binding sites (residue 286) matches that found after conjugation of annexin V with the far-red fluorophore Cy5.5 (32).

Biopotency of Annexin800

The biopotency of calcium-dependent, annexin800 binding to PS was quantified using surface plasmon resonance. As shown in Figure 3, annexin V and annexin800 (labeling ratio = 1.2) had similar binding capacities to PS. Indeed, the EC_{50} for calcium-dependence was 0.28 mM and 0.21 mM, for annexin V and annexin800, respectively. After 3 months of storage of annexin800 in the dark at 4°C, there was no change in potency (EC_{50} = 0.27 mM). Importantly, the fluorescence emission of annexin800 decreased only 8% over this same time (data not shown).

In vitro Cell Injury and Death Detection with Annexin800

In order to validate that annexin800 could be used to image cells, we induced cell death in U937 leukemic cells with etoposide and stained them using annexin800 (labeling ratio = 1.2 or 2.2). As shown in Figure 4, annexin800 was confirmed as a sensitive contrast agent for the detection of cell death, even with a labeling ratio of 2.2, although no attempt was made at isolating molecules with different degrees of substitution. In the absence of etoposide, basal annexin800 staining was present in $6.0 \pm 0.7\%$ and $5.6 \pm 0.8\%$ of cells stained using a fluorophore substitution ratio of 1.2 and 2.2, respectively. In the presence of etoposide, annexin800 staining was present in $87.9 \pm 8.0\%$ and $89.9 \pm 9.5\%$ of cells stained using a fluorophore substitution ratio of 1.2 and 2.2, respectively. The control protein HSA800 demonstrated no cell staining under any condition. All apoptotic cells identified by DAPI staining were also stained by annexin800 (data not shown).

Intraoperative Detection of Cell Injury and Death in the Rat Liver

To evaluate the efficacy of annexin800 to identify cell injury and/or death *in vivo*, we utilized three different I/R models; rat liver, rat intestine and canine heart. After 1 hr of ischemia and 2 hrs of reperfusion of the right lobes of the rat liver, annexin800 protein (labeling ratio = 1.2) was injected intravenously as described in Materials and Methods. Sufficient background clearance occurred within 10 min in the liver to provide the surgeon with optical imaging of cell injury and death (Figure 5A). Injection of the same concentration of CW800-CA resulted in no difference in dye accumulation between I/R and normal liver, no vascular leakage, and no evidence of infarction (data not shown). The SBR of the I/R liver increased immediately after injection of annexin800 and rose to 5.2 at 60 min, while the SBR of uninjured liver remained relatively constant at ≈ 2.0 and increased only slightly as background NIR fluorescence was cleared (Figure 5B).

Correlation of H&E, DAPI staining of all cell nuclei, TUNEL staining, and annexin800 NIR fluorescence (Figure 5C) revealed a strong correlation of annexin800 positivity of cell membranes with TUNEL positivity of cell nuclei. As might be expected, occasional cells were positive for one of the two markers, but not both. Annexin800 positivity without TUNEL positivity suggests reversible injury and/or early apoptosis, whereas TUNEL positivity without annexin800 positivity suggests either inaccessibility of the annexin800 to the cell (unlikely) or a late stage of apoptosis when cell membranes have already been phagocytosed.

Intraoperative Detection of Cell Injury and Death in the Rat Intestine

After 1 hr of ischemia and 2 hrs of reperfusion of a particular region of the small intestine, annexin800 protein (labeling ratio = 1.2) was injected intravenously. As shown in Figure 6A, annexin800 accumulated in the I/R intestine, but not in the normal intestine. The SBR of the I/R intestine increased immediately after injection of annexin800 and plateaued at 2.0 by 30 min, although that of the uninjured section remained constant at approximately 1.0 (Figure 6B).

Correlation of H&E, DAPI staining of all cell nuclei, TUNEL staining, and annexin800 NIR fluorescence is shown in Figure 6C. Although baseline TUNEL staining in the control intestine was expectedly high, there was no annexin800 positivity in these cells above the basement membrane, presumably since the intravenous injected agent did not have ready access to this compartment (data not shown). On the contrary, intestine subjected to I/R showed marked annexin800 positivity that correlated with TUNEL staining (Figure 6C).

Intraoperative Detection of Cell Injury and Death in Canine Heart

To assess the utility of intraoperative detection of cell injury and death with annexin800 in an animal approaching the size of humans, we performed an I/R model of canine heart. After 2 hrs of ischemia and 2 hrs of reperfusion, annexin800 (labeling ratio = 1.2) was injected intravenously. Adequate clearance had occurred by 30 min post-injection, and annexin800 accumulation distal to the occlusion could be seen (Figure 7A). After dissection of the heart, it was verified that annexin800 co-localized with TTC staining in the sub-endocardium, which represented the infarct area (Figure 7B). As is the case with rat experiments (Figure 5A), we observed prominent accumulation of annexin800 in the kidney, primarily to the renal cortex (Figure 7C).

DISCUSSION

The fluorescence emission of annexin800 has been optimized for 800 nm, a region of the optical spectrum with low tissue autofluorescence and relatively high tissue penetration (19), and fluorophore substitution has been titrated carefully to simultaneously maximize photon yield and biopotency (Figure 1C and Figure 3). Previous studies using annexin V labeled with visible fluorophores emitting at 525 nm (green) or 690 nm (far-red) demonstrated its usefulness in heart I/R and cancer models, respectively (16,33,34); however, autofluorescence, scatter, and photon penetration are far inferior to 800 nm (reviewed in (18)). Indeed, injury occurring below the surface of a large organ such as canine heart could still be seen on the surface using NIR fluorescent light (Figure 7), even with an annexin800 dose four-fold lower than that used in rat.

Blood clearance of annexin800 was rapid, with areas of injury becoming visible within 10 min, and peak SBR occurring within 30 min. Hence, annexin800 provides the surgeon with visual status of an entire tissue or organ in clinically realistic time frames. Injections can also be repeated as needed. One exception, though, is the kidney (35), where non-specific (36) accumulation is very high (Figure 7C), likely due to uptake of low-molecular-weight proteins by the proximal renal tubules. A recent study (27) suggests that changing the chemical structure of a Tc-99m chelator conjugated to radiolabeled annexin V can reduce liver and kidney uptake (at the expense of intestinal uptake); however, it is unknown at present if different NIR fluorophores will also differentially modulate annexin V biodistribution and clearance.

For organ transplantation, the technology we describe may prove useful during both procurement and post-transplant assessment. For procurement, annexin800 fluorescence could be used to ensure that donor organs are free from cellular damage. After transplantation and

reperfusion, annexin800 fluorescence could be used to quantify the extent of reperfusion injury in the entire organ. Future studies will focus on the quantification tools necessary to accomplish these latter two tasks. NIR fluorescent annexin V might also prove a useful agent for image-guided treatment of stroke and other pathological conditions associated with cell injury and death. Although a potential limitation of annexin V is that one cannot discriminate between reversible cell injury and death, since in both cases PS will be exposed on the surface of the cell, in surgical situations this might actually be an advantage. That is, annexin800 negativity would provide assurance that no cell injury or death has occurred, whereas quantification of annexin800 positivity, e.g., as a percent of total surface area, would alert the surgeon that damage has occurred. Importantly, since NIR light can penetrate relatively deeply into tissue, annexin800 provides sensitive detection of cellular injury even if the surface of the tissue or organ, as seen by the eye or with color video, appears normal (see for example, Figures 5A and 7A). However, it should be emphasized that scatter still limits the absolute depth penetration of NIR light, and that the image seen is exponentially weighted towards the surface rather than deep tissue (compare Figure 7A to 7B). Frequency-domain photon migration methods (37, 38), which can be incorporated into the present imaging system design, reduce this problem greatly, and suggest that depth sensitivity to 4 cm may someday be possible *in vivo*.

The key to the development of fluorescent proteins is the simultaneous optimization of total fluorescent yield and biopotency. Although it is usually possible to achieve high substitution ratios (Figure 1B), internal filter effects and dye quenching (30) limit total fluorescent yield (Figure 1C). As we have shown previously, wide spacing of heptamethine indocyanines over the surface of a large protein can minimize, but not eliminate these effects (25). Fluorophore substitution can also alter the molecule's biopotency, which needs to be verified independently. Previous studies found that annexin V substituted on residues 286 and 290 with Cy5.5 lost all bioactivity (32). However, we found that annexin800 substituted on residues 208 and 286 seem to maintain bioactivity (Figure 4). It is likely that steric hindrance and charge repulsion prevent the tetra-sulphonated indocyanines used in this study from conjugating so closely to each other, whereas the di-sulphonated indocyanines used previously are not as constrained. It is also possible that slight differences in pH between the two protocols favor certain sites over others. MALDI-TOF analysis revealed that our contrast agent is a mixture of various labeling ratios (Figure 1E). Although less likely based on its level of potency, it is possible that only the subpopulation of annexin800 substituted with a single fluorophore was bioactive.

In summary, we describe an 800 nm NIR fluorescent annexin V derivative that is optimized for total fluorescence yield, and which maintains biopotency. In conjunction with the appropriate imaging system, this molecule permits sensitive and specific intraoperative detection of cell injury and death in tissues and organs.

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A.

B.

C.

D.

Figure 1. Synthesis and Optical Properties of Annexin800

A) Absorbance of annexin800 (labeling ratio = 1.2) in PBS compared to 20% methanol (left ordinate). Fluorescence of annexin800 in 20% methanol (right ordinate).

B) The labeling ratio as a function of protein/fluorophore mixing ratio.

C) Per fluorophore fluorescence, as a function of labeling ratio, compared to free CW800-CA (1 μ M) in PBS (open circles). Per molecule total fluorescence yield calculated from the labeling ratio and per fluorophore fluorescence, and compared to the same dye concentration (1 μ M) of free CW800-CA in PBS (closed circles).

D) MALDI-TOF analysis of annexin800 (labeling ratio = 2.2) compared to annexin V.

Figure 2. Predicted Three-Dimensional Structure of Annexin800

The conjugation sites of CW800 to annexin V (white) were established using trypsin digestion and ES-TOF LC/MS peptide analysis, and are shown overlaid with the crystal structure (31). The amino acid substitution sites are Lys 208 (red) and Lys 286 (green). Annexin V's calcium binding sites are shown in yellow.

Figure 3. Biopotency of Annexin800

The biopotency of annexin V and annexin800 measured as the binding capacity to PS *in vitro* using plasmon surface resonance. EC_{50} of annexin V and annexin800 (labeling ratio = 1.2) is 0.28 mM and 0.21 mM, respectively. RU; resonance unit.

Figure 4. Detection of Cell Injury and Death *in Vitro*

U937 leukemic cells were treated with or without 50 μ M etoposide for 6 hrs, then stained with PBS (control) or 50 nM protein of either annexin800 (labeling ratio = 1.2), annexin800 (labeling ratio = 2.2), or CW800 conjugated to human serum albumin (HSA800; labeling ratio = 3.0).

A.

B.

C.

Figure 5. Detection of Cell Injury and Death after Ischemia/Reperfusion of Rat Liver

A) Right hepatic artery and corresponding portal vein were clamped for 1 hr, and reperused for 2 hrs. Imaging was performed at 30 min post-injection of 1.2 mg/kg of annexin800 protein (labeling ratio = 1.2; 40 nmol/kg of fluorophore total). I: ischemic segments; N: normal segments; Arrows mark right hepatic lobe subjected to I/R.

B) SBR (mean $\pm$ SEM; n = 6 animals) of ischemic versus normal liver post-injection of annexin800 as described in (A).

C) Frozen sections obtained from the liver in 5A were analyzed by H&E (top row), DAPI staining of all nuclei (second row), TUNEL staining (third row), and NIR fluorescence (bottom row). Shown are representative central (left) and portal (right) fields.

A.

B.

C.

Figure 6. Detection of Cell Injury and Death after Ischemia/Reperfusion of Rat Intestine

A) A branch artery of the superior mesenteric artery and its corresponding vein (arrowhead), and both sides of the occupying intestine (arrows) were clamped for 1 hr, and reperused for 2 hrs. Imaging was performed at 60 min post-injection of 1.2 mg/kg of annexin800 protein (labeling ratio = 1.2; 40 nmol/kg of fluorophore total).

B) SBR (mean $\pm$ SEM; n = 6 animals) of ischemic versus normal intestine post-injection of annexin800 as described in (A).

C) Frozen sections obtained from the intestine in 6A were analyzed by H&E (top row), DAPI staining of all nuclei (second row), TUNEL staining (third row), and NIR fluorescence (bottom row).

Figure 7. Detection of Cell Injury and Death after Ischemia/Reperfusion of Canine Heart

A) A branch of the left anterior descending artery (arrowhead) was occluded for 2 hrs, and reperused for 2 hrs. Imaging was performed at 30 min post-injection of 0.3 mg/kg of annexin800 protein (labeling ratio = 1.2; 10 nmol/kg of fluorophore total). Arrow marks territory at risk.

B) The heart was sectioned 60 min after injection of annexin800 and stained with TTC. NIR fluorescence was concordant with TTC staining (arrows).

C) The kidney was sectioned 60 min after injection of annexin800, revealing high annexin800 accumulation in the renal cortex.

TABLE 1

Mass Spectrometric Fingerprinting of CW800 Substitution Sites (boldface) of Annexin800

<u>Amino Acids</u>	<u>Sequence</u>	<u>Expected / Observed Mass</u>
208–212	KVFDK	636.8 / 637.3
286–290	KEFRK	579.7 / 579.3