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Video Article

Synthesis of an *In vivo* MRI-detectable Apoptosis Probe

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Abstract

Cellular apoptosis is a prominent feature of many diseases, and this programmed cell death typically occurs before clinical manifestations of disease are evident. A means to detect apoptosis in its earliest, reversible stages would afford a pre-clinical 'window' during which preventive or therapeutic measures could be taken to protect the heart from permanent damage. We present herein a simple and robust method to conjugate human Annexin V (ANX), which avidly binds to cells in the earliest, reversible stages of apoptosis, to superparamagnetic iron oxide (SPIO) nanoparticles, which serve as an MRI-detectable contrast agent. The conjugation method begins with an oxidation of the SPIO nanoparticles, which oxidizes carboxyl groups on the polysaccharide shell of SPIO. Purified ANX protein is then added in the setting of a sodium borate solution to facilitate covalent interaction of ANX with SPIO in a reducing buffer. A final reduction step with sodium borohydride is performed to complete the reduction, and then the reaction is quenched. Unconjugated ANX is removed from the mix by microcentrifuge filtration. The size and purity of the ANX-SPIO product is verified by dynamic light scattering (DLS). This method does not require addition to, or modification of, the polysaccharide SPIO shell, as opposed to cross-linked iron oxide particle conjugation methods or biotin-labeled nanoparticles. As a result, this method represents a simple, robust approach that may be extended to conjugation of other proteins of interest.

Video Link

The video component of this article can be found at http://www.jove.com/video/3775/

Protocol

Adapted from prior study[1].

1. Conjugation of Annexin V to SPIO

1. Oxidize SPIO particles (Ocean Nanotech Inc., 5 mg/mL) for 1 hr at 20 °C in the dark in solution with 0.15 M sodium periodate ($NaIO_4$) (4:1 weight:weight ratio)[2].
2. Incubate the oxidized SPIO for 12 hr with purified ANX protein (1:1 ratio, weight:weight) in 0.15 M sodium borate ($Na_2B_4O_7$ $10H_2O$) at 20 °C.
3. Reduce the mixture further for 1 hr with sodium borohydride ($NaBH^4$)under the fume hood and then quench with 0.15 M Tris-HCl.
4. Separate the free ANX from ANX-SPIO by centrifugation at 14,000 x g (75 µm pore Microcon filter, Millipore, Billerica, MA).
5. Further refine the compound by removing unbound impurities using a Magnetic Separator device (Ocean Nanotech, Inc.).
6. Quantify the filter retentate (ANX-SPIO) protein levels by Bradford protein assay and store at -80 °C at 2 mg/mL in 0.1 M Tris-HCl with 1% serum albumin and protease inhibitors (Halt Protease Inhibitor, Thermo Scientific, 1-50 mg/mg ANX).

2. Dynamic Light Scattering

1. Separately dilute the SPIO and ANX-SPIO in PBS to an optical density of 0.1 and analyze by dynamic light scattering in a Zetasizer Nano DLS machine (Malvern Inc., Worcestershire, UK)[3].
2. Obtain monomodal peaks (ideally) for the compound, with a Z-average particle size and polydispersity index (PDI). Compare this size to the DLS-measured size of the iron oxide nanoparticle alone. ANX-SPIO DLS results showed a conjugate particle hydrodynamic diameter of 78 nm with a PDI of 0.13[1], reflecting a homogenous ANX-SPIO species[3].

3. Cell Culture, Induction of Apoptosis in Culture, and MRI of Cultured Cells

1. Culture adult mouse bone marrow mesenchymal stem cells (mMSCs) in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (Invitrogen, Inc.), preferably at passage 20-30[4].
2. Induce apoptosis in the culture cells by treating for varying times at 37 °C with DOX (Sigma, 1 mM in 0.9% saline, 1 μM concentration in media)[12].
3. Quantify the amount of apoptosis in culture using either MRI with ANX-SPIO (described in 3.4 below) or using microscopy after TUNEL stain (APO-BrdUTM TUNEL Assay Kit, Molecular Probes/Invitrogen, Eugene, OR) or after stain with ANX-FITC (Roche, Switzerland).
4. Label the cells with ANX-SPIO (10 mg protein/mL medium except as noted), or with an equivalent weight of free SPIO (5 mg), for 15 min at 37 °C. Wash 4 times with PBS, and overlay with 1 mL of 1% agarose to prevent diffusion of the SPIO nanoparticles. Perform MRI of the dishes (described below). Alternatively, wash the cells and trypsinize after DOX and ANX-SPIO labeling, then allow the cells to form pellets in Eppendorf tubes.

4. *In Vitro* MRI

1. Use a 1.5 or 3 Tesla GE Signa EXCITE scanner with standard knee receiver coil for *in vitro* MRI analysis.
2. Place the culture dishes or tubes into an agar phantom, and image using a gradient-echo spoiled gradient sequence: Repetition Time (TR) 550 ms, Echo Time (TE) 10 ms, flip angle 15°, matrix 256 x 256, field-of-view 3 cm x 3 cm, slice thickness 1.3 mm, number of averages (NEX) 2, spacing 0 mm[5]. Image axially through the cell layer on the plate for T2* signal loss, using regions-of-interest (ROIs) of a fixed area (for instance, 0.8 mm^2). Adjust for background using control ROI signal from the agar phantom. Measure the background noise from the signal of the air surrounding the phantom.
3. Calculate the MRI Contrast-to-Noise Ratio (CNR: [SI sample ROI-SI control ROI]/[SD of SI of background noise], where SI is signal intensity, and SD is standard deviation) was calculated for each culture dish.

5. Representative Results

Measurement of T2* signal loss *in vitro* requires a homogeneous agar phantom and the lack of air bubbles, which will create signal void artifact. This artifact is difficult to distinguish from genuine T2* signal loss of SPIO, and is critical to interpretable results. Please see **Figure 1** for an example of an air artifact as well as genuine iron oxide T2* signal loss.

For *in vitro* culture dish imaging, apply an appropriate volume of agar gel to each culture dish, so that sufficient axial MRI slices, which are approximately 1 mm thick, can be made to adequately image the entire dish. **Figure 2** illustrates the expected MRI signal from individual culture plates in agar. There is typically some signal variation at the edges of the plate, so analysis of regions of interest should avoid these uneven areas.

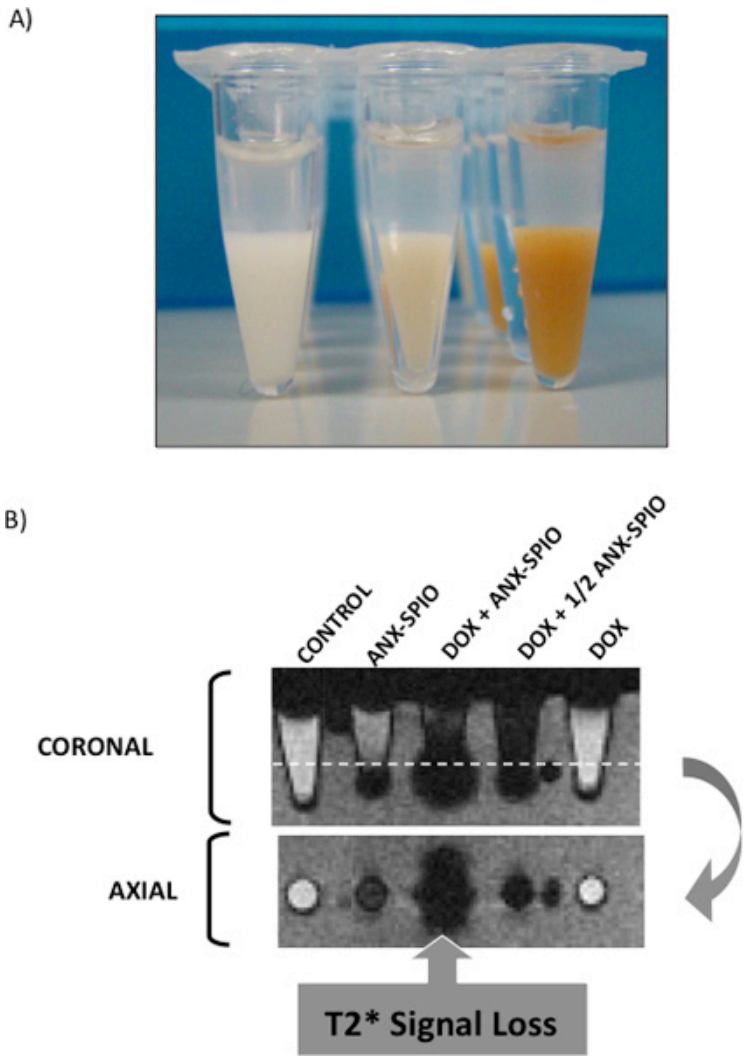

Figure 1. ANX-SPIO detects apoptosis in cultured neonatal rat cardiomyocytes. **Figure 1A** shows a photo of pelleted neonatal cardiomyocytes treated with control solution (left tube), ANX-SPIO (middle tube), and DOX+ANX-SPIO (right tube). Note the brownish color of the DOX+ANX-SPIO treated cells, reflecting increased retention of the ANX-SPIO compound in apoptotic cells. **Figure 1B** *In vitro* MRI images show the intense T2* signal loss of the DOX+ANX-SPIO cells. Also, an air bubble is seen between the 4th and 5th Eppendorf tubes (small black signal void), which is similar to the signal void of ANX-SPIO, and which can affect analysis of CNR.

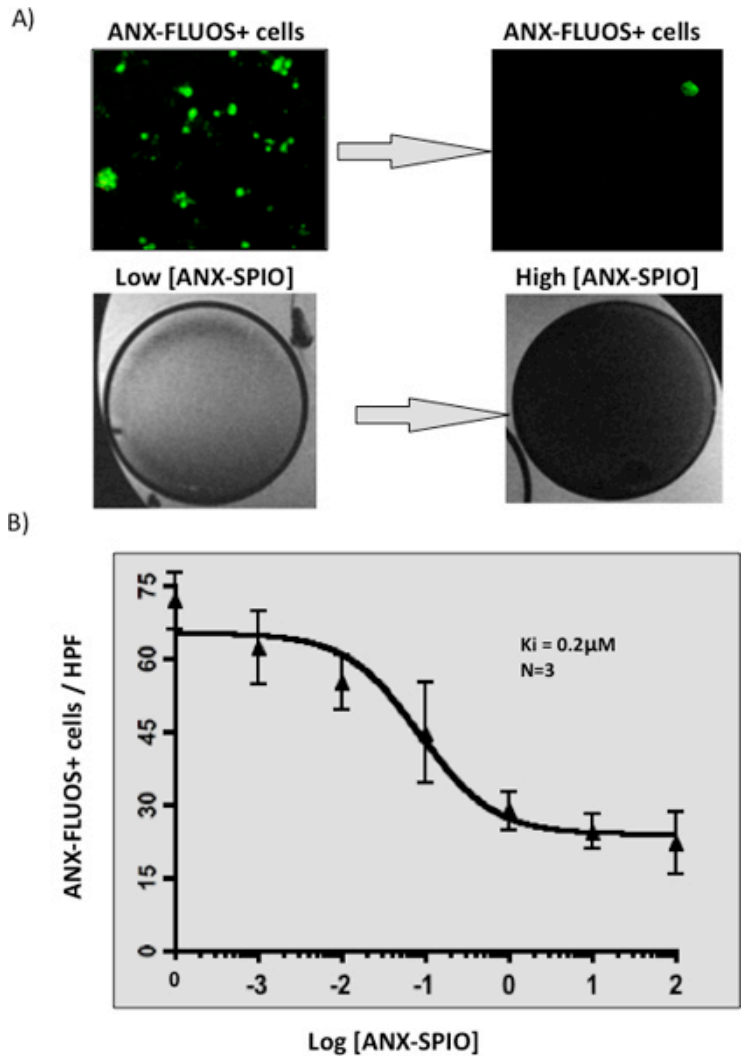

Figure 2. ANX-SPIO binding is specific for apoptotic cells in culture. **Figure 2A** demonstrates apoptotic rat neonatal cardiomyocytes on culture dishes, subjected to either ANX-FLUOS staining or ANX-SPIO staining, with low and high concentrations (left and right, respectively) of ANX-SPIO. Note the different T2* signal loss (black signal) of apoptotic cells that were treated with fluorescent ANX (ANX-FLUOS) and low levels versus high levels of ANX-SPIO. The increased T2* signal loss on the right is associated with a markedly reduced ANX-FLUOS signal, which has been competed off by higher ASX-SPIO concentrations. **Figure 2B** illustrates the competitive binding of ANX-SPIO versus ANX-FLUOS signal and the dissociation constant, K_i. The X-axis denotes the Annexin protein concentration in log scale.

Movie 1. Click here to view supplemental movie.

Discussion

The described conjugation method for linking SPIO to Annexin V exploits the side chain amine groups of Annexin and the carboxyl moieties of the SPIO nanoparticle. Through specific oxidation-reduction steps, covalent linkage of these compounds can be achieved, and the resulting functionalized nanoparticle can be isolated. This method may be generalized to other proteins and nanoparticles of interest.

The most critical steps are the oxidation and reduction conditions, carried out in the appropriate temperatures and with gentle mixing. Low yield may result from incomplete oxidation or reduction, which results in residual free Annexin V that is removed during the filtration step. When applied to other proteins of interest, optimal conditions may vary from those described above. However, once those optimal conditions are defined, the filtered free protein may decrease to a negligible amount, and therefore, microcentrifuge filtration may become superfluous. Indeed, excess filtration may reduce final compound yield due to nonspecific binding to the filter itself. If this occurs, pre-filtering 1% albumin through the filter will reduce nonspecific binding of the conjugated compound to the filter and improve the yield. DLS analysis of the final compound, and well as protein concentration measurements of the filtrate, will help determine if residual free protein is present.

The presence of free SPIO in the final compound is also a potential contaminant. To this end, DLS was employed to check purity. A monomodal peak by DLS helps to ensure a singular iron oxide species. Free SPIO alone produces a distinct DLS peak at 46nm and was not present in ANX-SPIO preparations.

Several options for bioconjugation are available[6-12] that employ the alternative conjugating mechanisms, such as biotin-streptavidin interaction or protein cross-linking of customized nanoparticles. The method presented herein represents a robust, widely applicable approach for conjugation of magnetic nanoparticles to proteins of interest, without the use of specialized nanoparticles.

Disclosures

No conflicts of interest declared.

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