# Annexin A5 conjugated quantum dots with a paramagnetic lipidic coating for the multimodal detection of apoptotic cells

G. A. van Tilborg[1], W. J. Mulder[1], P. T. Chin[2], C. P. Reutelingsperger[3], G. J. Strijkers[1], K. Nicolay[1]

[1]Biomedical NMR, Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, Netherlands, [2]Molecular Materials and Nanoscience, Department of Chemistry, Eindhoven University of Technology, Eindhoven, Netherlands, [3]Cardiovascular Research Institute of Maastricht, Department of Biochemistry, Maastricht University, Maastricht, Netherlands

## Introduction

Apoptosis, or programmed cell death, is an important feature of normal tissue development and homeostasis. Deregulation of the apoptotic program may result in a variety of diseases, including cancer, atherosclerosis and myocardial infarction[1]. Therefore the apoptotic pathway appears to be a promising target for therapeutic purposes. Consequently the visualization of apoptosis could be of great importance for the early detection of therapy efficiency and the evaluation of disease progression. The exposure of phosphatidylserine (PS) at the outer monolayer of the plasma membrane of apoptotic cells[2] is an important hallmark of apoptosis that is often used for the visualization of apoptosis. For this purpose the protein annexin A5, which binds with high affinity to this negatively charged phospholipid in the presence of $Ca^{2+}$, is conjugated to a contrast agent for the imaging modality of choice. MRI allows the non-invasive detection of MR contrast agents in high spatial resolution anatomical images through the complete living animal. However, MRI is a relatively insensitive technique and it lacks the microscopic resolution of the optical methods. Therefore the bimodal approach of using MRI in combination with optical imaging would greatly enhance the ability to visualize apoptosis in vivo. For optimal detection with both imaging techniques a bimodal annexin A5-conjugated contrast agent was designed, which is composed of a quantum dot with a paramagnetic lipidic coating.

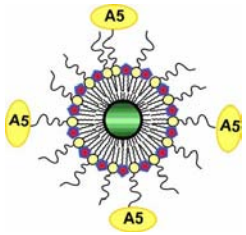


Figure 1: Schematic representation of a quantum dot with a paramagnetic lipidic coating to which multiple annexin A5 proteins are conjugated.

## Materials and methods

CdSe/ZnS core/shell quantum dots were synthesized under a flow of argon by injection of the precursors into a hot coordinating solvent. The TOPO/HDA capped quantum dots were coated with paramagnetic micelles according to an extended procedure described by Dubertret et al[3]. Micelles were composed of PEG-DSPE, Mal-PEG-DSPE, and the paramagnetic lipid Gd-DTPA-BSA. Following preparation empty micelles and micelles containing a quantum dot were separated with ultracentrifugation. Subsequently, recombinant annexin A5, containing a single free cystein, was covalently linked to the lipid-coated quantum dots by sulfhydryl-maleimide coupling. The resulting nanoparticle was approximately 15 nm in size and the ionic relaxivities at 37 °C and 60 MHz, were $r_1 = 12.4 \pm 0.4$ and $r_1 = 18.0 \pm 0.1$ ($mM^{-1}s^{-1}$). Nanoparticles without Gd-lipid had negligible ionic relaxivities. To assess the specificity of this contrast agent for apoptotic cells the nanoparticles were incubated for 30 minutes in a 2.5 mM $Ca^{2+}$ containing binding buffer with viable Jurkat cells (***3***) or with Jurkat cells that had been pre-incubated with anti-Fas (***4***) in order to induce apoptosis. Cells that were not incubated with contrast agents were used as a control (***1***). Since annexin A5 binds to phosphatidylserine in a $Ca^{2+}$-dependent manner, the removal of $Ca^{2+}$ will prevent binding. Therefore, annexin A5 conjugated nanoparticles that were incubated in a buffer containing EDTA were also used as a control (***2***). After extensive washing in either 2.5 mM $Ca^{2+}$ or 5 mM EDTA containing buffer the cells were fixed in a 4% paraformaldehyde solution, containing either $Ca^{2+}$ or EDTA. Subsequently the cells were allowed to settle as loosely packed pellets overnight in 250 µl tubes. Cell pellets of the different incubations were placed in UV light to confirm the presence of annexin A5-quantum dots (anxA5-QDs) within the cell pellets. Additional fluorescence microscopy was performed for spatial localization of the contrast agents. $T_2$- and $T_1$-weighted spin-echo images of the cell pellets were obtained and $T_2$ and $T_1$ relaxation times were quantified at 6.3T. One should note that the fraction of apoptotic cells within the viable cell culture (***3***) might be increased due to the incubation and washing procedures.

| | anti-Fas | anxA5 QDs | $Ca^{2+}$ | EDTA |
|---|---|---|---|---|
| **1** | - | - | + | - |
| **2** | + | + | - | + |
| **3** | - | + | + | - |
| **4** | + | + | + | - |

Table 1: Cell incubation conditions.

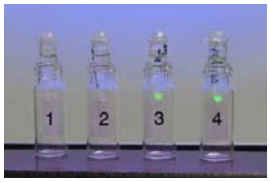


Figure 2: Fixed cell pellets were placed in 254 nm UV light.

## Results

An overview of all incubation conditions of cells with the annexin A5-QDs (Fig1) is given in Table 1. The numbering in Table 1 is used in all figures. Illumination of the different cell pellets with 254 nm UV light shows two green emitting cell pellets (Fig2) that can be clearly distinguished from the other cell pellets. The green emitting light originates from viable- or apoptotic cells that were incubated with annexin A5-nanoparticles in the presence of $Ca^{2+}$. Fluorescence microscopy of a small diluted sample of the cell pellets showed massive association of the annexin A5-QDs to apoptotic cells, and to a smaller fraction of the cells within the viable cell sample (Fig3). These cell pellets also displayed decreased signal intensity in $T_2$-weighted images or increased signal intensity in $T_1$-weighted images, compared to the controls (Fig4). On the other hand little association of annexin A5-nanoparticles was observed in the absence of $Ca^{2+}$ both with fluorescence microscopy (Fig3) and MRI (Fig4). $T_2$ and $T_1$ values of the cell pellets were expressed as relaxation rates $R_2$ ($T_2^{-1}$) and $R_1$ ($T_1^{-1}$). Both $R_2$ and $R_1$ were significantly higher for the pellets of viable or apoptotic cells incubated with annexin A5-QDs in the presence of $Ca^{2+}$ than the two control cell pellets (Fig5). In addition annexin A5-nanoparticles were shown to significantly increase $R_2$ and $R_1$ values of apoptotic cells compared to viable cells (Fig5).

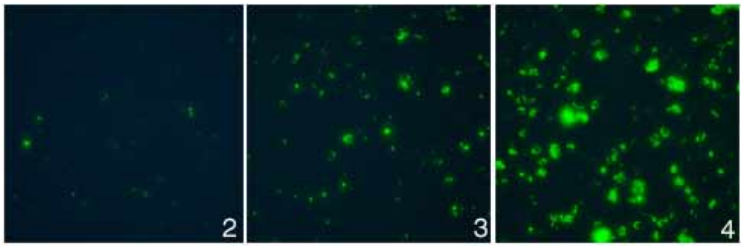


Figure 3: Fluorescence microscopy of fixed cells, 365 nm excitation (20x).

## Conclusion

We have developed a novel nanoparticulate contrast agent for the detection of apoptosis, with excellent paramagnetic and fluorescent properties. The in vitro results demonstrated a high specificity of this contrast agent for apoptotic cells, confirming its potential for the detection of apoptosis in different animal models with both intravital fluorescence microscopy and MRI in vivo.

**References**

1. Haunstetter A.*et al*, *CircRes.*, 15;82(11):1111-1129 (1998).
2. Fadok V.A.*et al.*, *Nature*, 4;405(6782):85-90 (2000).
3. Dubertret B. *et al. Science,* (298):1759-1762 (2002).

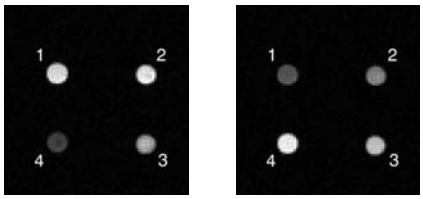


Figure 4: $T_2$-weighted, TR=2s/TE=20ms (left) and $T_1$-weighted image, TR=150ms/TE=8.8ms (right) of fixed cell pellets.

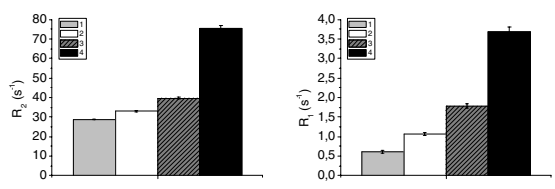


Figure 5: $R_2$ relaxation rates (A) and $R_1$ relaxation rates (B) of cell pellets. Error bars represent the error on the relaxation rates from the fitting-procedures.