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Cell-Permeable and Biocompatible Polymeric Nanoparticles for Apoptosis Imaging

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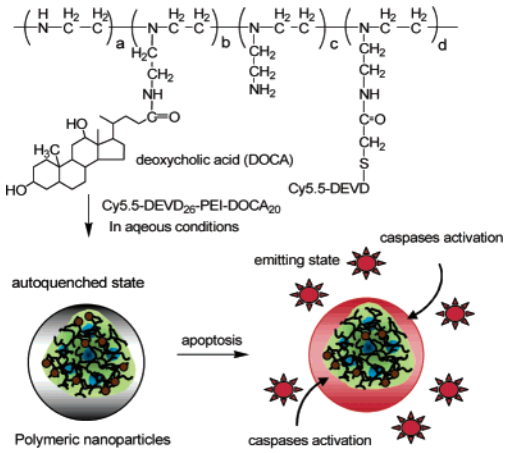
Apoptosis is a normal physiological process that occurs during embryonic development and participates in the maintenance of tissue homeostasis.[1] Improper regulation of apoptosis can lead to several pathological conditions, including cancer and neurodegenerative diseases. Measurement of annexin V binding allows the imaging of an early event in apoptosis, namely, the cell surface exposure of aminophospholipids, which normally reside on the cytoplasmic leaflet of the plasma membrane.[2] However, cell surface binding of annexin V is not completely specific to apoptosis; it is also found in some normal and necrotic cells. Thus, an apoptosis-specific probe is urgently needed for the early diagnosis of various diseases and the discovery and evaluation of new apoptosis-related drugs.

Herein, we synthesized and characterized cell-permeable and biocompatible polymeric nanoparticles that are specifically activated by effector caspases of caspase-3 and caspase-7 because the activation of these effector caspases is a crucial event in the process of apoptosis in many cell types.[3] Of note, these cysteine proteases of caspase-3 and caspase-7 cleave specifically the N-terminus of tetra motif *Asp-Glu-Val-Asp* (DEVD). This tetrapeptide motif of DEVD has been used to develop caspase-specific probes for apoptosis imaging at the single-cell level.[4]

Using polymer-based nanotechnology, we attached the effector caspase-specific near-infrared (NIR) fluorescence probe, Cy5.5-DEVD, to amphiphilic bile acid-modified polymer backbone, which can be designed to form an autoquenched polymeric nanoparticle structure (Scheme 1). This polymeric nanoparticle can be cell-permeable, biocompatible, and allow the proximity of Cy5.5-DEVD molecules to be easily controlled. Similar probes containing autoquenched far-red fluorochromes have been developed for a variety of proteases, including caspase-1, cathepsin D, and matrix metalloproteases, and these protease-activatable imaging probes presented a high fluorescent signal amplification after expose to the target protease.[5]

Cell-permeable and biocompatible polymer,[6] branched poly(ethyleneimine) (PEI, M_w = 25 kDa), was modified with deoxycholic acid (DOCA) hydroxysuccinimide ester, resulting in PEI-DOCA nanoparticles. A TNBS assay showed that PEI-DOCA had an average molecular weight of 32.5 kDa, indicating that it contained 20 ± 1.8 DOCA molecules (PEI-DOCA$_{20}$). Next, we iodoacetylated the amine groups of PEI-DOCA$_{20}$ nanoparticles with iodoacetic anhydride to introduce thiol-specific reactivity.[5b] The caspase-specific probe, Cy5.5-peptide-SH, was chemically linked to the iodoacetylated PEI-DOCA$_{20}$ in aqueous conditions. The Cy5.5-peptide-SH was prepared by linking hydroxysuccinimide

Scheme 1. Schematic Diagram of a Cell-Permeable and Biocompatible Polymeric Nanoparticle for Apoptosis Imaging



Cy5.5 to the N-terminus of the caspase-cleavable peptide (DEVD) or the control noncleavable peptide (DEVG) (Table 1).[3] By TNBS assay, each PEI-DOCA$_{20}$ polymer contained 26 ± 3.2 molecules of Cy5.5-DEVD (Cy5.5-DEVD$_{26}$-PEI-DOCA$_{20}$) or 25 ± 2.2 molecules of Cy5.5-DEVG (Cy5.5-DEVG$_{25}$-PEI-DOCA$_{20}$), respectively. Under optical reaction conditions, polymer conjugates form a polymeric nanoparticle in which the fluorescence probes, Cy5.5-DEVDs, are maximally autoquenched.

Dynamic light scattering and TEM results showed that the freshly prepared Cy5.5-DEVD$_{26}$-PEI-DOCA$_{20}$ was essentially spherical and approximately 80−100 nm in diameter (Figure 1a). NIR fluorescence imaging showed that the autoquenched Cy5.5-DEVD molecules in Cy5.5-DEVD$_{26}$-PEI-DOCA$_{20}$ were readily cleaved by both recombinant caspase-3 and caspase-7 (Figure 1b), but not by other caspases, such as caspase-6 and caspase-9 (see Supporting Information Figure SI2). In the presence of both caspase-3 and caspase-7, the Cy5.5-DEVD$_{26}$-PEI-DOCA$_{20}$ solution revealed an approximately 10- or 7-fold increase in fluorescent intensity compared to background, confirming that 60−86% of the fluorescent intensity of autoquenched Cy5.5 is recovered by the enzyme activation. However, the cleavage of Cy5.5-DEVD$_{26}$-PEI-DOCA$_{20}$ to both effector caspases was inhibited completely by 10 mM of the caspase-3 inhibitor, Ac-DEVD-CHO. As a control experiment, the fluorescence intensity of Cy5.5-DEVG$_{25}$-PEI-DOCA$_{20}$ did not change in the presence of both caspase-3 and caspase-7 because the control peptide DEVG cannot be cleaved by both proteases.

Imaging probe entry into cells is an important area in apoptosis imaging because the caspases' reaction occurs in the cytoplasm. Thus, we tracked the fluorescein isothiocyanate (FITC)-labeled Cy5.5-DEVD$_{26}$-PEI-DOCA$_{20}$ nanoparticles in HeLa cells

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Table 1. Caspase-Specific and Control Peptide Sequences

name	sequence
DEVD	Gly-Gly-*Asp*-*Glu*-*Val*-*Asp*-Gly-Gly-Cys
DEVG	Gly-Gly-*Asp*-*Glu*-*Val*-*Gly*-Gly-Gly-Cys

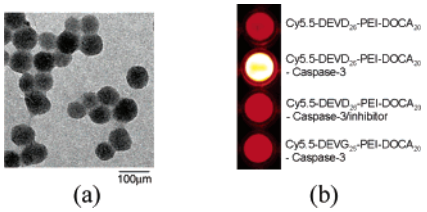

Figure 1. (a) TEM image of polymeric nanoparticles in aqueous conditions. (b) NIR fluorescence imaging of a 96-well containing polymeric nanoparticles with/without caspase-3 or with caspase-3 inhibitor (Ac-DEVD-CHO), respectively.

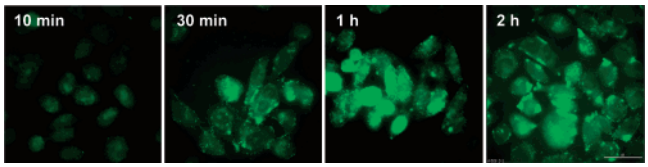

Figure 2. Cellular uptake of polymeric nanoparticles as a function of incubation time. HeLa cells (5×10^3) incubated with FITC-labeled Cy5.5-$DEVD_{26}$-PEI-$DOCA_{20}$ (10 μg/mL).

(Figure 2). With increasing incubation time, the FITC fluorescence increased, reaching a maximum at 1 h. Moreover, the internalized polymer nanoparticles were mainly localized in the cytoplasm, but some particles were observed in the perinuclear compartment (see 3D Z scanning image in Figure SI3). To assess the biocompatibility of Cy5.5-$DEVD_{26}$-PEI-$DOCA_{20}$, we examined its effect on cell viability (see Supporting Information Figure SI4). At concentrations up to 20 μg/mL, Cy5.5-$DEVD_{26}$-PEI-$DOCA_{20}$ was not cytotoxic. On the basis of the biocompatibility test, we determined that the optimal concentration of Cy5.5-$DEVD_{26}$-PEI-$DOCA_{20}$ is 10 μg/mL, a concentration at which the bioavailability is 100% for 2 days.

To confirm that polymeric nanoparticles can be used to visualize caspase-dependent apoptosis, we preincubated HeLa cells with nanoparticles and then treated them with tumor necrosis factor-related apoptosis, inducing ligand (TRAIL) (50 ng/mL), which is a potent inducer of apoptosis in HeLa cells.[7] The caspase-3 activity of TRAIL-treated HeLa cells was measured with immunoblotting and the synthetic fluorogenic caspase-3 substrate Ac-DEVD-AMC (see Supporting Information Figure SI5). It should be noted that nanoparticles alone did not appear to activate caspase-3 in normal cells. After 30 min of TRAIL treatment, however, caspase-3 activity increased, with maximal activity (6.4-fold activation) obtained after 1 h.

Finally, we used NIR fluorescence microscopy to examine the ability of nanoparticles to visualize caspase activation in TRAIL-treated HeLa cells (Figure 3). Prior to treatment with TRAIL, HeLa cells incubated with nanoparticles did not show any NIR fluorescent signal, and this low background of nanoparticles in live cells can allow real-time imaging of apoptosis. After a 10 min treatment with TRAIL, there is no NIR fluorescent signal. However, after a 30 min treatment with TRAIL, we observed a strong NIR fluorescent

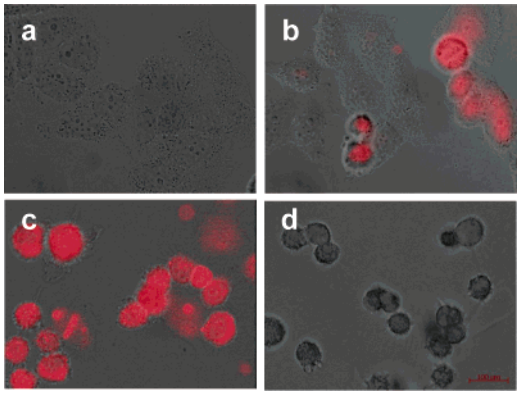

Figure 3. NIR fluorescence microscopy of HeLa cells incubated with Cy5.5-$DEVD_{26}$-PEI-$DOCA_{20}$ (10 μg/mL) for (a) 10 min, (b) 30 min, and (c) 1 h in the presence of TRAIL (50 ng/mL), and (d) the control Cy5.5-$DEVG_{25}$-PEI-$DOCA_{20}$ (10 μg/mL) was also incubated for 2 h in the presence of TRAIL (50 ng/mL).

signal with 12-fold fluorescent intensity, compared to background. Interestingly, phase contrast microscopy revealed that cells showing strong NIR fluorescent signals shrank and formed membrane blebs, changes characteristic of cells undergoing apoptosis. After a 1 h treatment with TRAIL, all of cells showed strong NIR fluorescence signals and signs of apoptosis displaying apoptotic body. However, the treated control nanoparticle cells, Cy5.5-$DEVG_{25}$-PEI-$DOCA_{20}$, did not present any NIR fluorescent signal even after a 2 h treatment with TRAIL, confirming the high specificity of the autoquenched polymer nanoparticles for active caspases (Figure 3d).

In summary, we developed a cell-permeable, biocompatible, and autoquenched imaging probe, polymeric nanoparticle that is capable of real-time imaging of apoptosis in living cells. This polymeric nanoparticle was efficiently and specifically cleaved by both caspase-3 and caspase-7 in cell-free condition and cell culture system. This polymeric nanoparticle can be used to measure apoptosis in cell-based high-throughput screens for inhibitors or inducers of apoptosis.

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Supporting Information Available: Detailed experimental procedures and background on the polymeric nanoparticles. This material is available free of charge via the Internet at http://pubs.acs.org.

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