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Quantum Dots Based Probes Conjugated to Annexin V for Photostable Apoptosis Detection and Imaging

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ABSTRACT

Quantum dots (Qdots) are nanoparticles exhibiting fluorescent properties that can be used for cell staining. We present here the development of quantum dots conjugated to Annexin V for specific targeting of apoptotic cells, for both apoptosis detection and staining of apoptotic "living" cells. For that purpose, Qdots Streptavidin Conjugates are coupled to biotinylated Annexin V, a 35-kDa protein which specifically recognizes and binds to phosphatidylserine (PS) moieties present on the outer membrane of apoptotic cells and not on healthy or necrotic cells. By using Annexin V, our Qdots probes are made specific for apoptotic cells. Staining of apoptotic cells was checked using fluorescence and confocal microscopy techniques and nonfixed cells. It is shown here that Qdots are insensitive to bleaching after prolonged exposure as opposed to organic dyes. This makes Qdots excellent candidates to continuously follow fast changes occurring at the membrane of apoptotic cells and facilitates time-lapse imaging as they alleviate any bleaching issue.

Nanotechnologies and nanomaterials are currently gaining enormous popularity in the fields of biological applications.[1,2] In particular, quantum dots (Qdots) are emerging as a revolutionary means for imaging and optical detection.[3−6] This may be accounted for by the unique physical, chemical, and optical properties[7] of these nanocrystals based on semiconductor materials (e.g., CdSe@ZnS Qdots). Compared to organic dyes, Qdots exhibit a series of advantages which make them particularly attractive for staining applications. First, they present higher extinction coefficients[8] and higher quantum yields[9] and they are less prone to photobleaching.[3,10] Second, their absorbance properties as well as their emission wavelength depend on the size of the semiconductor core, which can be finely tuned.[7,11] Their excitation window is broad and is the same for any Qdot whereas the emission window is rather narrow (∼20 nm),[12] making them particularly suitable for multiplexed staining. More than six Qdots could be used simultaneously in the same assay with no or minimal interference among each other.[7,13] Finally, their toxicity should be limited compared to that of organic dyes, although this point is still under controversy.[8,14−16] All these reasons make them very good candidates for fluorescent staining of cells. Indeed, live cell imaging and time-lapse imaging, which are highly relevant for cellular studies, require photostable dyes and use multiple targets. Qdots seem therefore to be logical candidates for such applications and consist of a promising alternative to fluorescent dyes for the study of complex processes in cells. Many papers have already reported on the use of quantum dots for cell staining or tracking.[4,17,18] Qdots have been used for instance for whole cell staining after their coupling to a specific peptidic signal[19,20] or using various means for internalization,[21] or for specific staining of organelles, e.g., mitochondria or nuclei[22,23] as well as the cell membrane.[24,25] Single molecules have also been tracked using Qdots staining.[26−29] Finally, Qdots have been applied for in vivo imaging.[4,8,30,31]

Apoptosis is an example of biological phenomena that involve many subprocesses. Apoptosis consists of a programmed or suicidal cell death.[32,33] It takes place in tissues as a means to regulate cell number by counterbalancing cell proliferation.[33] Dysfunction in apoptosis is therefore linked to disease states[33] which are correlated either to a lack of or to an excess of cell suicides. Understanding processes underlying apoptosis will help to find new targets for drug development and curing diseases.[34]

Apoptosis involves changes at various levels in the cells and at different time frames, and this cascade of events can be divided into a succession of three phases: initiation,

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decision, execution. This complex process is triggered by a series of enzymes named caspases (decision phase) after activation of a death receptor (initiation phase). Execution consists of various changes in the cells. First, cells change at the mitochondrion level. Subsequently, changes occur on the outer membrane, e.g., changes in membrane mechanical and electrical properties, cell shrinkage, and redistribution of phospholipids between the inner and the outer membrane. For instance, phosphatidylserine groups (PS), which are normally located on the inner membrane, are externalized. At a later stage, the nuclear membrane is disrupted, and DNA fragments and chromatin condensate. At the end, apoptotic bodies containing the remains from the cell are produced. A complete picture of apoptotis can be obtained by the study of multiple cellular targets and through time-lapse imaging to follow changes over time. In this context, organic dyes present limitations because of their limited photostability and tendency for bleaching and due to the restricted number of dyes that can be used simultaneously without any spectral interference. Imaging of apoptotic "living" cells typically uses a combination of several dyes targeting different organelles and reflecting changes at various sublevels in the cell.[35] For instance, changes at the mitochondrion level can be detected with specific dyes,[36] membrane changes can be imaged with dyes showing the externalization of PS moieties,[37] and DNA staining shows the permeabilization of the nucleus membrane and chromatin condensation.[38]

We focus here on an early and major event in the apoptotic cascade, the externalization of phosphatidylserine (PS) occurring through the "flipping" of the cell membrane.[39] Targeting PS moieties consist of one of the main methods to detect apoptotic cells and to isolate them.[40] Other methods rely on the detection of DNA fragmentation and on the permeabilization of the nuclear membrane. However, PS externalization precedes any change in nucleus,[41] and moreover it is specific of apoptosis and does not occur in the case of necrosis, the other form of cell death. Consequently, numerous kits exist for apoptosis studies and/or detection target PS moieties sitting on the outer membrane of apoptotic cells. Two main agents enable the specific recognition of PS moieties. A small protein, Annexin V (35 kDa), which binds to PS in the presence of Ca^{2+},[42] is the most widely used agent. Zn-based ligands that interact with phospholipid moieties[43,44] have also been developed. In both cases, the recognition agent is coupled to a second part which can be detected or trapped (optical detection, radiolabeling, magnetic particles for cell sorting, immunodetection, ...).[45−48]

We report here on Qdots functionalized with Annexin V for detecting and staining apoptotic "living" cells through specific recognition of PS moieties located on the outer membrane of the cells. For that purpose we decided to exploit the well-known streptavidin−biotin interaction, by coupling biotinylated Annexin to Qdots Streptavidin Conjugates. This coupling is very strong even if it is not covalent and is commonly used in biology. It is easy and fast, does not require any sophisticated chemistry, and can be readily achieved starting from commercially available products (biotinylated Annexin V, Qdots Streptavidin Conjugates). Apoptotic cells which were stained with Annexin V-functionalized Qdots and counterstained with usual organic dyes conjugated to Annexin V such as FITC− or Alexa Fluor 647−Annexin V conjugates were imaged using confocal microscopy. We particularly demonstrated the superiority of Qdots toward organic dyes with respect to bleaching issues. Thereby, they appear more suitable for time-lapse imaging of living cells undergoing the process of apoptosis. Moreover their photostability allows for more frequent imaging, which enables the visualization of fast events such as those occurring at the membrane level.

Two approaches were concomitantly investigated (see Figure 1): (i) Qdots were first functionalized with Annexin V and subsequently added to apoptotic cells, or (ii) apoptotic cells were preincubated with Annexin V, and Qdots were added later once Annexin V had bound to PS moieties.

Typically, cells were first treated overnight with an inducer of apoptosis (e.g., etoposide at 50 μM or camptothecin at 6 μM). The medium was then changed to a medium enriched with calcium (2.5 mM) to enable binding of Annexin V to PS, and staining agents were added, quantum dots and organic dyes, either in one step or in two steps. After 60 min of incubation (37 °C, 5% CO_2), cells were imaged by confocal microscopy.

The ratio of Annexin V molecules to Qdots was determined using data from the manufacturer. Qdots Streptavidin Conjugates present a functionalization level of 5−10 streptavidin molecules/Qdot, and we decided to functionalize Qdots with approximately two molecules of biotinylated Annexin V. This corresponds to 1 μL (∼4 pmol) of the commercially available solution of biotinylated Annexin V (assessed concentration of 3.6 μM) and 2 pmol of Qdots Streptavidin Conjugates. Typically, for 0.5 mL of cells in suspension (approximately 0.5×10^6 cells), we used 2 pmol of Qdots Streptavidin Conjugates and 1 μL (∼4 pmol) of biotinylated Annexin V (final concentrations of 4 and 8 nM, respectively).

It should be noted that our Qdots are prone to aggregate in solution if no care is taken, and thereby they do not efficiently stain the cells. Actually, the Qdots used for this work are not coated with PEG (poly(ethylene glycol)), the latter aiming at alleviating any aggregation issue and any unspecific penetration into cells. Therefore, Qdots were dissolved in PBS 1× buffer, the solution was vortexed, and only the supernatant was added to the cells; this was seen to improve the situation and to limit aggregation. We also assume that Qdots may self-aggregate around Annexin V molecules as each Annexin V is coupled to three to four biotin residues; subsequently, Annexin V no longer is recognized by PS moieties or interacts with them.

We decided to simultaneously investigate another approach to prevent such aggregation from occurring. Apoptotic cells were preincubated with biotinylated Annexin V, and subsequently Qdots were subsequently added once Annexin V was assumed to be on the cell surface (for instance, when cells were positive to a fluorescent dye conjugated with Annexin V). For this procedure, the Annexin V:Qdots ratio was changed to 1:1. Typically, we used 2 pmol of biotinylated Annexin V and 2 pmol of Qdots Streptavidin Conju-

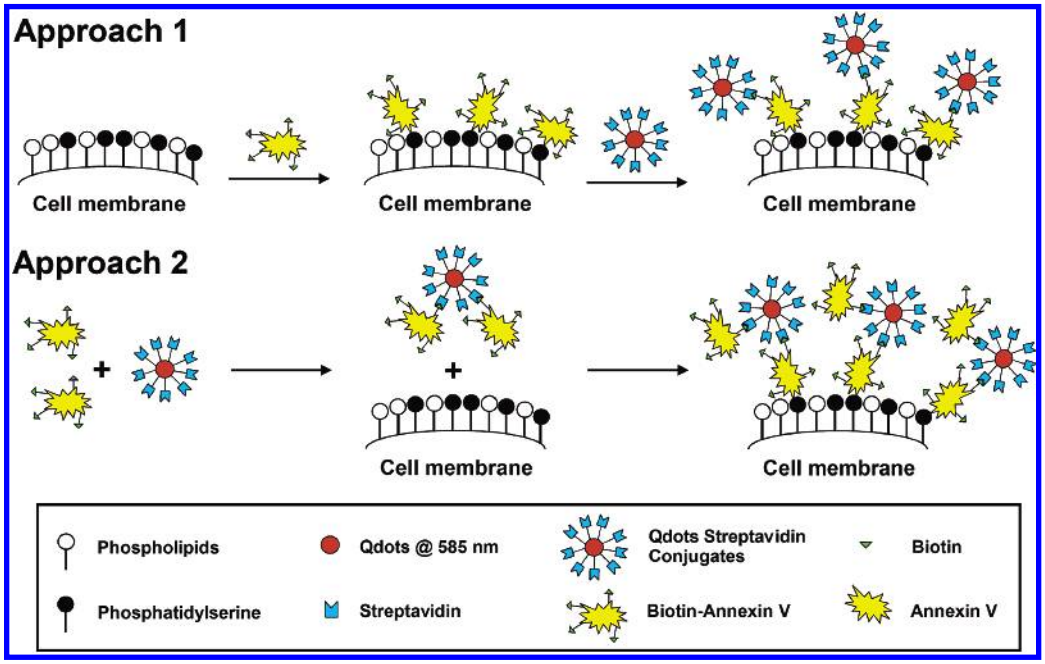

Figure 1. Two approaches used for staining apoptotic cells with Annexin V-coupled Qdots. (Approach 1) Preincubation of apoptotic cells with biotinylated Annexin V in a 2.5 mM $CaCl_2$ medium followed by addition of Qdots Strepatvidin Conjugates. (Approach 2) Preparation of Annexin V-functionalized Qdots subsequently added to cells.

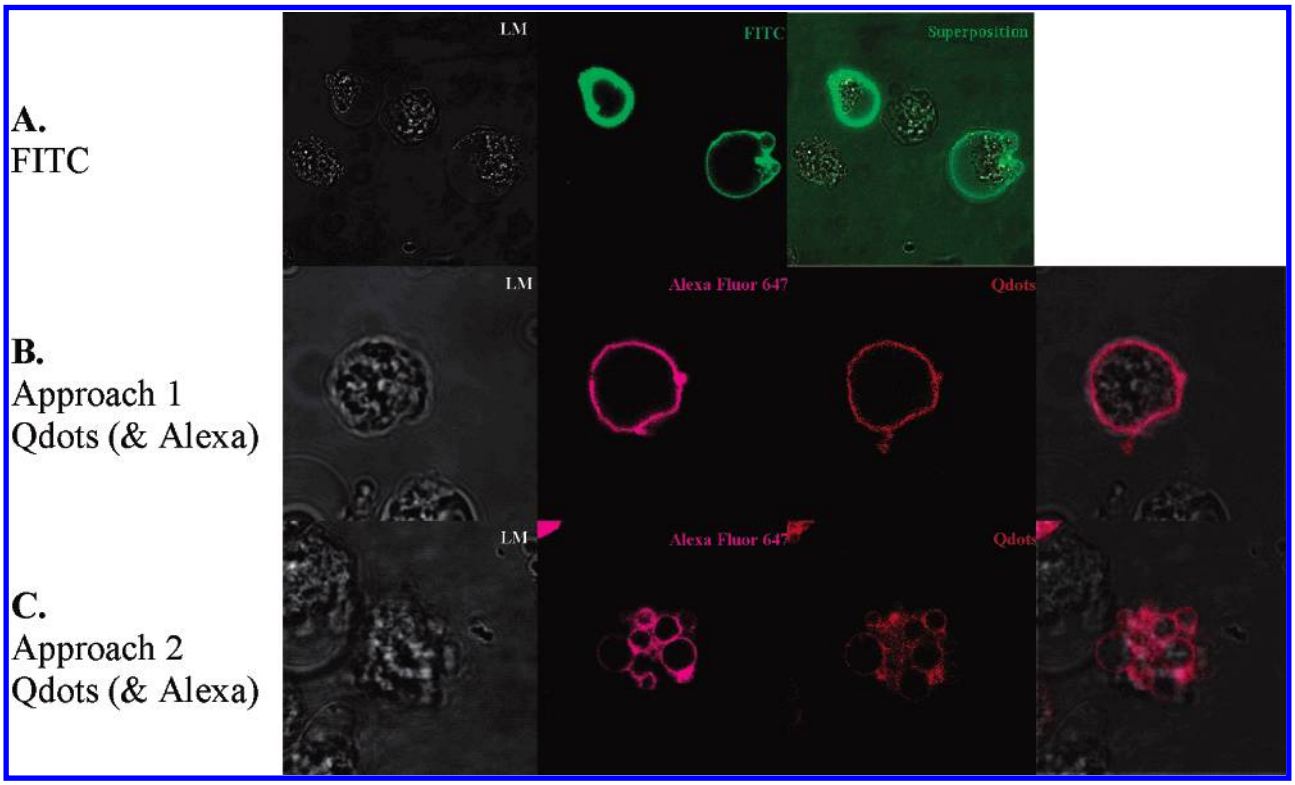

Figure 2. Staining patterns of apoptotic cell using (A) FITC−Annexin V (0.5% v/v, green) and (B, C) both Alexa Fluor 647 (0.5% v/v, pink) and Annexin V-functionalized Qdots (red). Qdots staining achieved either in two steps (preincubation of apoptotic cells with biotinylated Annexin V in a 2.5 mM $CaCl_2$ medium followed by addition of Qdots Strepatvidin Conjugates; 4 nM Qdots and 4 nM Annexin V) (B) or in one step (preparation of Annexin V-functionalized Qdots subsequently added to cells; 4 nM Qdots and 8 nM Annexin V) (C).

gates for 500 μL of cells in suspension. After 60 min of incubation, cells were imaged.

In both cases, apoptotic HL 60 cells were stained with Annexin V-functionalized Qdots and the staining pattern was consistent with a specific PS binding, i.e., a crownlike pattern on the outer membrane of the apoptotic cell, as is the case when using organic dyes (Figure 2). Cell staining achieved by using Qdots was compared to results obtained by using organic dyes such as FITC− or Alexa Fluor 647−Annexin V conjugates (0.5% v/v). For that purpose, apoptotic cells

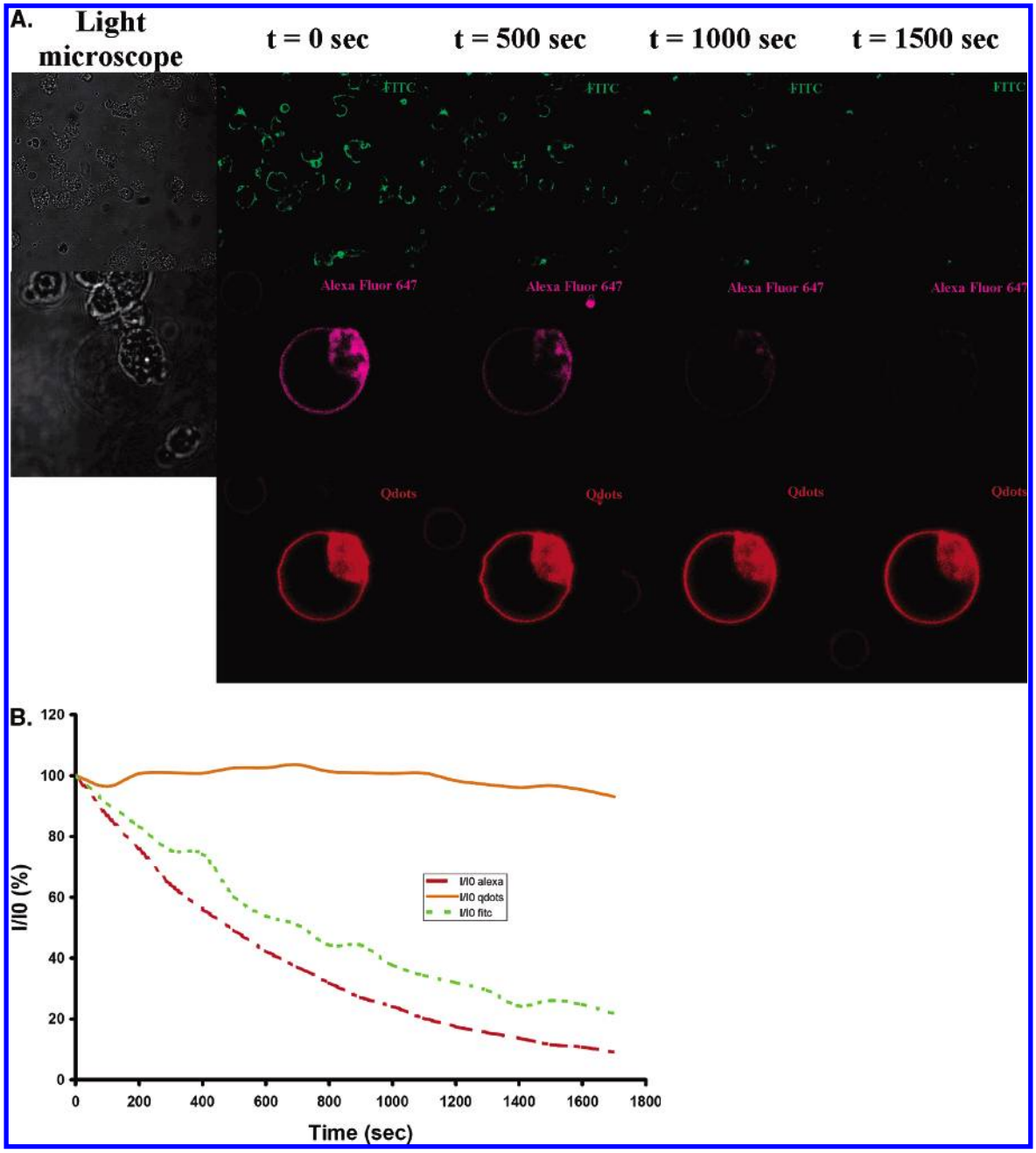

Figure 3. Comparison of the staining efficiency of Annexin V-functionalized Qdots (staining achieved in one step using 2 nM of Qdots and 4 nM of biotinylated Annexin V, red), FITC−Annexin V (0.5% v/, green) and Alexa Fluor 647−Annexin V (0.5% v/v, pink). Laser intensity set at 4% for FITC excitation and pictures taken every 6 s; laser intensity set at 10% and 20% for Alexa Fluor 647 and Qdots, respectively, and pictures taken every 10 s. (A) Light microscope pictures and pictures taken at $t = 0$, 500, 1000, 1500 s. (B) Intensity variation as a function of time for the three staining agents conjugated to Annexin V, FITC, Alexa Fluor 647 and Qdots. Intensity measured and integrated on a whole cell using ImageJ software. Intensity values expressed as relative values I/I_0, where I_0 is the intensity measured at the first irradiation of the sample.

were costained with Annexin V-functionalized Qdots and an Annexin V-conjugated organic dye (e.g., Alexa Fluor 647 or FITC). The matching of both staining profiles confirms that our Annexin V-functionalized Qdots bind indeed to PS moieties on the outer membrane of the cell. Furthermore, Qdots remain on cells after two steps of washing with fresh medium. Figure 2 illustrates the staining of apoptotic cells in various conditions, FITC− or Alexa Fluor 647−Annexin V conjugates, Qdots with a preincubation with Annexin V (Figure 2B) and Annexin-V functionalized Qdots (Figure 2C). Moreover, control experiments were also carried out on healthy HL 60 cells (without any induction of apoptosis); no parasitic staining of cells was observed, which demonstrates the specificity of these Annexin V-functionalized Qdots as is the case of fluorescent dyes.

To further illustrate the potentiality of our Annexin V-functionalized Qdots, we diversified the means for inducing apoptosis; several drugs were tested (50 μM etoposide, 4 μM camptothecin (CPT), 3 nM TNF-alpha and 50 μM cycloheximide (CHX)) as well as UV irradiation of cells (5

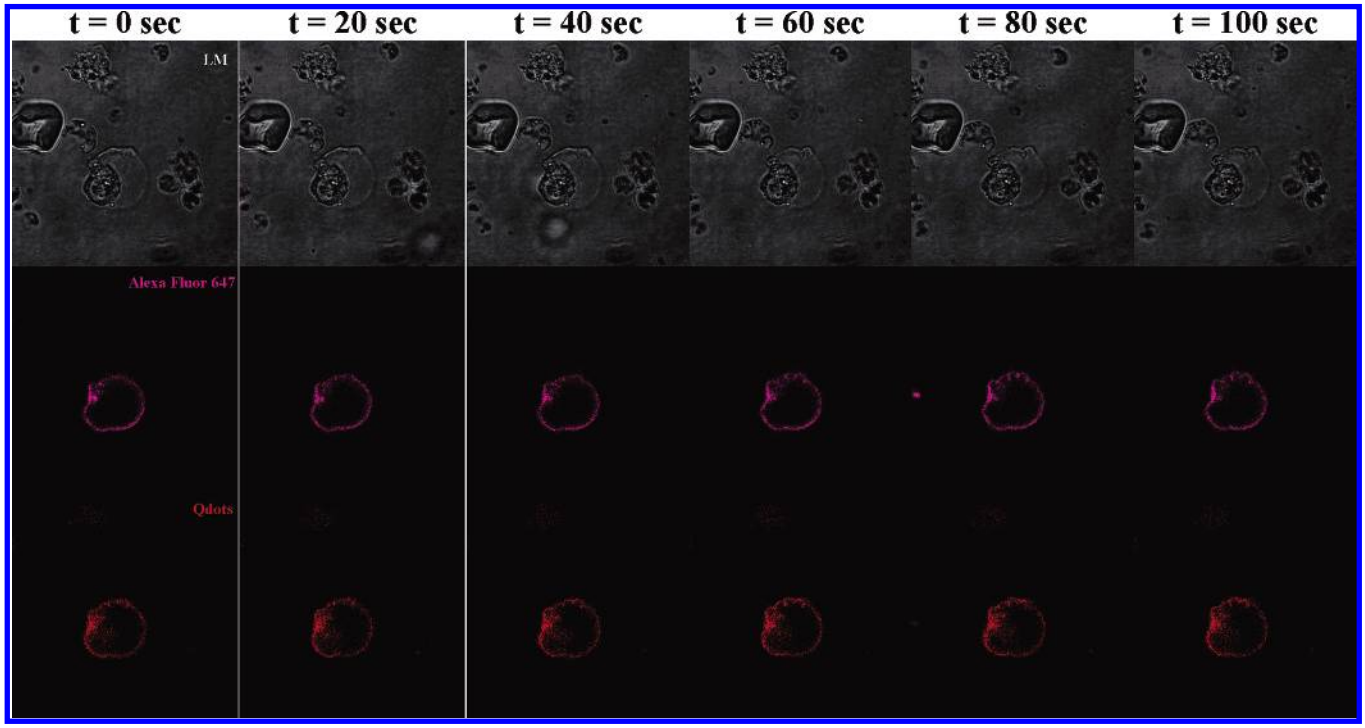

Figure 4. Changes at the membrane level occurring in a few minutes. Sequences of pictures illustrating the blebbing of the membrane and taken every 10 s over a period of 200 s. Light microscope picture and fluorescence pictures resulting from both Qdots staining (red) and Alexa Fluor 647 counterstaining (pink) taken at $t = 0$, 20, 40, 60, 80, 100, 120, 140, and 200 s.

min at $\lambda = 254$ nm). Another cell type was also assessed; RAW 264.7 macrophages were cultivated for 2 days in appropriate chamber plates for microscopy studies, treated with, e.g., 6 μm CPT, and Annexin V-functionalized Qdots were added to cells in a $CaCl_2$ 2.5 mM buffer. In every case, apoptotic cells were stained with Annexin V-functionalized Qdots (data not shown).

We finally compared Annexin V-functionalized Qdots to standard organic dyes for time-lapse imaging of apoptotic "living" cells, i.e., in terms of staining efficiency and photostability. Two organic dyes were tested here, FITC−and Alexa Fluor 647−Annexin V conjugates. The former one is known to bleach very quickly upon laser or mercury lamp excitation whereas the latter one presents higher photostability. When organic dyes were used, cells could not be imaged for a long period of time. In the case of FITC, bleaching occurs within 15−20 min (pictures taken every 6 s) with a laser intensity set at 4% (see Figure 3A). Alexa Fluor 647 was seen to be more resistant to laser irradiation, but after 25 min of imaging (pictures taken every 10 s, laser intensity was set at 10%), Qdots still stained the cells whereas Alexa Fluor 647 had fully bleached (see Figure 3A). It should be noted that these studies were carried out using standard image resolution, with an average pixel time of 1 μs and images of 512 × 512 pixels. Figure 4 represents the variation of the intensity over time for a prolonged irradiation of cells stained with either Annexin V-functionalized Qdots or Annexin V-conjugated organic dyes (FITC or Alexa Fluor 647 conjugated to Annexin V). The intensity of Qdots does not decrease, whereas the emission of both organic dyes becomes very low after less than 100 pictures (see Figure 3B). It should be noted that this bleaching issue is particularly of importance for tracking very fast processes during a long period of time. In the present case, once a cell has entered the apoptotic cascade, ensuing events, e.g., membrane blebbing and PS externalization, happen very fast, even within less than 5 min depending on the cell line and the inducer. When organic dyes are used, due to this bleaching issue and the limited amount of pictures that can be taken, pictures must be spaced from 4 to 5 min, and when apoptosis occurs, only a few pictures illustrate the process. Moreover, high-resolution pictures (longer pixel time up to 6.4 μs and higher pixel number up to 1024 × 1024 pixels) must be avoided as it would mean longer exposure times to the laser light. For instance, with such a temporal space, events such as membrane flipping and blebbing cannot be followed and imaged in time, as their time scale is shorter than the imaging frequency. Figure 4 illustrates the changes at the membrane level on an apoptotic cell, which happen in less than 2 min. Pictures were taken every 10 s for that purpose. Using an organic dye, it is not possible to follow the whole process: fluorescent dyes (e.g., Alexa Fluor 647) bleach too fast and too much. On the contrary, at the end of the experiment, Qdots still stained the sample and the membrane of the cell undergoing apoptosis without any decrease in intensity.

From these positive results one can imagine several applications of such Annexin V-conjugated Qdots. We have focused here on one particular application that consists of imaging "living" cells undergoing apoptosis; using Qdots enables not only frequent but also high-resolution imaging of cells as it alleviates any bleaching issues. Thereby it is possible to follow the time-course of the process of apoptosis. For instance, cell response delay can be imaged after application of the stimulus to induce apoptosis; this could

be achieved using a double staining based on Qdots at the single cell level. Using Qdots could make possible dose−response studies on apoptotic cells, for (i) studying the onset delay of apoptosis depending on the drug or inducer dose and for (ii) the quantification of PS at the surface of apoptotic cells. This may be easier to perform using fixed cells and not "living" cells. Kinetics studies can also be carried out on fixed cells, the process of apoptosis being stopped at given time values and cells being subsequently stained.

It should be noted indeed noted that our Annexin V-conjugated Qdots are rather large, especially compared to their organic counterparts, as they comprise two layers of proteins, one layer of 5−10 streptavidin molecules and one layer of 1−2 Annexin V molecules. The size of the resulting conjugated Qdots (∼20 nm) limits the diffusion time toward PS moieties. Therefore, real time studies of apoptosis onset may be hindered if using these Qdots, especially if working with "living" cells for deeper biological studies as the diffusion issue may hinder "real-time" studies. However a direct coupling of Annexin V on Qdots should limit this diffusion issue and facilitate kinetics studies on "living" cells.

Conclusion. In this paper we have presented a novel method for photostable detection and imaging of apoptotic cells using Qdots. These quantum dots are functionalized with Annexin V proteins and target the PS moieties which are externalized during the apoptosis process and which are located on the outer membrane of apoptotic cells. The preparation of Annexin V-functionalized Qdots was successfully achieved using a streptavidin−biotin interaction, starting from commercially available products. Apoptotic cells, treated with CHX (50 μM) and TNF-alpha (3 nM), CPT (4 μM), etoposide (50 μM), or UV irradiation (λ = 254 nm) for 5 min, were successfully stained with Annexin V-conjugated Qdots using two procedures. In the first one, cells are preincubated with biotinylated Annexin V and Qdots Streptavidin were successively added, whereas in the second one, Qdots were first functionalized with Annexin V and then added to apoptotic cells. Both methods gave comparable results, similar to those of conventional staining techniques (organic dyes). Thereby we have demonstrated the efficiency of our Qdots functionalized with Annexin V that can specifically recognize and stain apoptotic cells. Moreover we have illustrated the usefulness of Qdots photostability over periods of several hours as opposed to organic dyes which suffer from photobleaching in around 30 min. Photostability of staining agents enable more frequent imaging of cells; thereby fast events such as those occurring at the membrane of apoptotic cells can be visualized. With organic dyes, which are prone to bleaching phenomena, such events would be missed.

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Supporting Information Available: Experimental details including reagents, cell culture, cell staining, preparation of Qdots, and confocal microscopy. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Whitesides, G. M. *Small* **2005**, *1* (2), 172−179.
(2) Niemeyer, C. M. *Angew. Chem., Int. Ed.* **2001**, *40* (22), 4128−4158.
(3) Jaiswal, J. K.; Mattoussi, H.; Mauro, J. M.; Simon, S. M. *Nat. Biotechnol.* **2003**, *21* (1), 47−51.
(4) Michalet, X.; Pinaud, F. F.; Bentolila, L. A.; Tsay, J. M.; Doose, S.; Li, J. J.; Sundaresan, G.; Wu, A. M.; Gambhir, S. S.; Weiss, S. *Science* **2005**, *307* (5709), 538−544.
(5) Parak, W. J.; Pellegrino, T.; Plank, C. *Nanotechnology* **2005**, *16* (2), R9−R25.
(6) Medintz, I. L.; Uyeda, H. T.; Goldman, E. R.; Mattoussi, H. *Nat. Mater.* **2005**, *4*(6), 435−446.
(7) Michalet, X.; Pinaud, F.; Lacoste, T. D.; Dahan, M.; Bruchez, M. P.; Alivisatos, A. P.; Weiss, S. *Single Mol.* **2001**, *2* (4), 261−276.
(8) Dubertret, B.; Skourides, P.; Norris, D. J.; Noireaux, V.; Brivanlou, A. H.; Libchaber, A. *Science* **2002**, *298* (5599), 1759−1762.
(9) Peng, Z. A.; Peng, X. G. *J. Am. Chem. Soc.* **2001**, *123* (1), 183−184.
(10) Dahan, M.; Laurence, T.; Schumacher, A.; Chemla, D. S.; Alivisatos, A. P.; Sauer, M.; Weiss, S. *Biophys. J.* **2000**, *78* (1), 385A−385A.
(11) Alivisatos, A. P. *J. Phys. Chem.* **1996**, *100* (31), 13226−13239.
(12) Gerion, D.; Pinaud, F.; Williams, S. C.; Parak, W. J.; Zanchet, D.; Weiss, S.; Alivisatos, A. P. *J. Phys. Chem. B* **2001**, *105* (37), 8861−8871.
(13) Lacoste, T. D.; Michalet, X.; Pinaud, F.; Chemla, D. S.; Alivisatos, A. P.; Weiss, S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97* (17), 9461−9466.
(14) Tsay, J. M.; Michalet, X. *Chem. Biol.* **2005**, *12* (11), 1159−1161.
(15) Kirchner, C.; Liedl, T.; Kudera, S.; Pellegrino, T.; Javier, A. M.; Gaub, H. E.; Stolzle, S.; Fertig, N.; Parak, W. J. *Nano Lett.* **2005**, *5* (2), 331−338.
(16) Derfus, A. M.; Chan, W. C. W.; Bhatia, S. N. *Nano Lett.* **2004**, *4* (1), 11−18.
(17) Pellegrino, T.; Parak, W. J.; Boudreau, R.; Le Gros, M. A.; Gerion, D.; Alivisatos, A. P.; Larabell, C. A. *Differentiation* **2003**, *71* (9−10), 542−548.
(18) Parak, W. J.; Boudreau, R.; Le Gros, M.; Gerion, D.; Zanchet, D.; Micheel, C. M.; Williams, S. C.; Alivisatos, A. P.; Larabell, C. *Adv. Mater.* **2002**, *14* (12), 882−885.
(19) Mattheakis, L. C.; Dias, J. M.; Choi, Y. J.; Gong, J.; Bruchez, M. P.; Liu, J. Q.; Wang, E. *Anal. Biochem.* **2004**, *327* (2), 200−208.
(20) Lagerholm, B. C.; Wang, M. M.; Ernst, L. A.; Ly, D. H.; Liu, H. J.; Bruchez, M. P.; Waggoner, A. S. *Nano Lett.* **2004**, *4* (10), 2019−2022.
(21) Derfus, A. M.; Chan, W. C. W.; Bhatia, S. N. *Adv. Mater.* **2004**, *16* (12), 961+.
(22) Chen, F. Q.; Gerion, D. *Nano Lett.* **2004**, *4* (10), 1827−1832.
(23) Hoshino, A.; Fujioka, K.; Oku, T.; Nakamura, S.; Suga, M.; Yamaguchi, Y.; Suzuki, K.; Yasuhara, M.; Yamamoto, K. *Microbiol. Immunol.* **2004**, *48* (12), 985−994.
(24) Alexson, D.; Li, Y.; Ramadurai, D.; Shi, P.; George, L.; George, L.; Uddin, M.; Thomas, P.; Rufo, S.; Dutta, M.; Stroscio, M. A. *IEEE Trans. Nanotechnol.* **2004**, *3* (1), 86−92.
(25) Sukhanova, A.; Devy, M.; Venteo, L.; Kaplan, H.; Artemyev, M.; Oleinikov, V.; Klinov, D.; Pluot, M.; Cohen, J. H. M.; Nabiev, I. *Anal. Biochem.* **2004**, *324* (1), 60−67.
(26) Mansson, A.; Sundberg, M.; Balaz, M.; Bunk, R.; Nicholls, I. A.; Omling, P.; Tagerud, S.; Montelius, L. *Biochem. Biophys. Res. Commun.* **2004**, *314* (2), 529−534.
(27) Lidke, D. S.; Nagy, P.; Heintzmann, R.; Arndt-Jovin, D. J.; Post, J. N.; Grecco, H. E.; Jares-Erijman, E. A.; Jovin, T. M. *Nat. Biotechnol.* **2004**, *22* (2), 198−203.
(28) Wu, X. Y.; Liu, H. J.; Liu, J. Q.; Haley, K. N.; Treadway, J. A.; Larson, J. P.; Ge, N. F.; Peale, F.; Bruchez, M. P. *Nat. Biotechnol.* **2003**, *21* (1), 41−46.
(29) Dahan, M.; Levi, S.; Luccardini, C.; Rostaing, P.; Riveau, B.; Triller, A. *Science* **2003**, *302* (5644), 442−445.

(30) Gao, X. H.; Cui, Y. Y.; Levenson, R. M.; Chung, L. W. K.; Nie, S. M. *Nat. Biotechnol.* **2004**, *22* (8), 969−976.
(31) Akerman, M. E.; Chan, W. C. W.; Laakkonen, P.; Bhatia, S. N.; Ruoslahti, E. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99* (20), 12617−12621.
(32) Green, D. R.; Evan, G. I. *Cancer Cell* **2002**, *1* (1), 19−30.
(33) Vermes, I.; Haanen, C. Apoptosis And Programmed Cell-Death In Health And Disease. In *Advances In Clinical Chemistry*; 1994; Vol. 31, pp 177−246.
(34) Kerr, J. F. R.; Winterford, C. M.; Harmon, B. V. *Cancer* **1994**, *73* (8), 2013−2026.
(35) Munoz-Pinedo, C.; Green, D. R.; van den Berg, A. *Lab-on-a-Chip* **2005**, *5* (6), 628−633.
(36) Vayssiere, J. L.; Petit, P. X.; Risler, Y.; Mignotte, B. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91* (24), 11752−11756.
(37) Vermes, I.; Haanen, C.; Steffensnakken, H.; Reutelingsperger, C. *J. Immunol. Methods* **1995**, *184* (1), 39−51.
(38) Plantin-Carrenard, E.; Bringuier, A.; Derappe, C.; Pichon, J.; Guillot, R.; Bernard, M.; Foglietti, M. J.; Feldmann, G.; Aubery, M.; Braut-Boucher, F. *Cell Biol. Toxicol.* **2003**, *19* (2), 121−133.
(39) Fadok, V. A.; Voelker, D. R.; Campbell, P. A.; Cohen, J. J.; Bratton, D. L.; Henson, P. M. *J. Immunol.* **1992**, *148* (7), 2207−2216.
(40) van Engeland, M.; Nieland, L. J. W.; Ramaekers, F. C. S.; Schutte, B.; Reutelingsperger, C. P. M. *Cytometry* **1998**, *31* (1), 1−9.
(41) Martin, S. J.; Reutelingsperger, C. P. M.; McGahon, A. J.; Rader, J. A.; Vanschie, R.; Laface, D. M.; Green, D. R. *J. Exp. Med.* **1995**, *182* (5), 1545−1556.
(42) Andree, H. A. M.; Reutelingsperger, C. P. M.; Hauptmann, R.; Hemker, H. C.; Hermens, W. T.; Willems, G. M. *J. Biol. Chem.* **1990**, *265* (9), 4923−4928.
(43) Hanshaw, R. G.; Lakshmi, C.; Lambert, T. N.; Johnson, J. R.; Smith, B. D. *ChemBioChem* **2005**, *6* (12), 2214−2220.
(44) Kimura, E.; Aoki, S.; Kikuta, E.; Koike, T. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (7), 3731−3736.
(45) Koopman, G.; Reutelingsperger, C. P. M.; Kuijten, G. A. M.; Keehnen, R. M. J.; Pals, S. T.; Vanoers, M. H. J. *Blood* **1994**, *84* (5), 1415−1420.
(46) Wang, G. P.; Song, E. Q.; Xie, H. Y.; Zhang, Z. L.; Tian, Z. Q.; Zuo, C.; Pang, D. W.; Wu, D. C.; Shi, Y. B. *Chem. Commun.* **2005**, *34*, 4276−4278.
(47) Vermes, I.; Haanen, C.; Reutelingsperger, C. *Clin. Chem.* **1995**, *41* (6), S91−S91.
(48) Engbers-Buijtenhuijs, P.; Kamphuis, M.; Veer, G. V.; Haanen, C.; Poot, A. A.; Feijen, J.; Vermes, I. *Apoptosis* **2005**, *10* (2), 429−437.

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