Detection of dying cells using lectin-conjugated fluorescent and luminescent nanoparticles

Erkennung von sterbenden Zellen mit Hilfe von Phytagglutinin – Verbindung fluoreszierender und lumineszierender Nanopartikel

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1 Introduction

There has been a great deal of interest in the fabrication and characterization of highly luminescent and fluorescent nanoparticles (NPs) because of their potential applications as biosensors in molecular diagnostics. The organic dyes currently used in biolabeling have advantages such as commercial availability and high quantum yield, but they also have disadvantages such as a broad spectral band, a short fluorescence lifetime and photobleaching. In contrast, rare-earth nanoparticles exhibit sharp emission bands, reasonably large Stokes shifts and long fluorescence lifetimes, and they can be encapsulated in neutral environments to avoid toxic reactions. In addition, rare-earth nanoparticles have a high quantum yield and excellent photostability [1].

Detection of pathological cells that are dying by apoptotic or necrotic pathways is of great importance for clinical diagnostics. Since anomalous expression of these cells creates a significant danger for the organism, causing a development of autoimmune disorders or inflammation, correspondingly, detection and binding of apoptotic and necrotic cells for their subsequent removal from the organism are of potential therapeutic value [2].

Necrosis is a passive, catabolic, pathological cell death process which generally occurs in response to external toxic factors such as inflammation, ischemic or toxic injury. During the programmed cell death (also named as apoptosis) cell performs a suicide accompanied by enzymatic slicing intracellular content, including proteins and DNA. As a result, cell is shrinking and fragmented into membrane-coated vesicles called apoptotic bodies which are engulfed by cells of the immune system. Many biological tests were proposed for apoptosis detection. Most of them are based on measuring changes in specific biochemical components of the dying cell. However, since these changes occur inside the cell, its integrity should be violated. Thus, the results of such analysis may not correspond to what is really going on in the apoptotic cell.

In our previous studies we screened cell surface in order to find new biochemical markers of apoptosis and necrosis which could be accessible for analysis without cell destruction. We found that glycoprotein pattern of apoptotic cells is changed significantly, namely an increase in the exposure levels of α-D-mannose and β-D-galactose-rich glycoproteins and a decrease in the exposure levels of sialic-rich glycoproteins were revealed [3–7]. We utilized various lectins (proteins that are capable of binding specific carbohydrates or carbohdrate moiety of glycoprotein molecule) as a tool for detection of cells undergoing apoptosis and necrosis.

In this work, we conjugated lectins, specifically recognizing cells dying by apoptosis and necrosis, to the surface of luminescent GaN:Eu³⁺ nanoparticles providing a green and red fluorescence depending on excitation wavelength and polystyrene nanoparticles containing high amount of fluorescein (0.5 %) in their core (fluoro-NPs) and thus providing bright and stable green fluorescence. Both type of nanoparticles were covered with polymeric carbon chain of functional surface-active peroxides (SAP) bearing reactive epoxy groups with the aim to isolate the fluorescent core and allow conjugation of specific proteins to the nanoparticle surface. The developed lectin-conjugated NPs were effective in detection of both apoptotic and necrotic cells providing a bright and stable signal.

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2 Materials and Methods

The GaN:Eu³⁺ nanoparticles have been synthesized by the combustion method with some modifications. The details of the samples preparation have been already described by us elsewhere [8].

Synthesis of fluorescein-containing polystyrene nanoparticles was performed by Dr. Zaichenko and his group, at Lviv Polytechnical University by co-polymerization of fluorescein molecules with polystyrene (Provisional US Patent, 20080626).

Nanoparticles were covered with polymeric carbon chain of functional surface-active peroxides (SAP) bearing reactive epoxy groups as described previously in our work [9].

Conjugation of lectin to SAP-covered nanoparticles was performed as following: protein was dissolved in 0.1M NaHCO₃, pH 8.0 (final concentration 1–2 mg/ml) and added to previously sonicated (44 kHz, 4x90 seconds) suspension of nanoparticles, covered with SAP, and incubated for 2 hours at 37°C under active mixing. To stop the reaction Tris-HCl, pH 7.5, final concentration 0.15 M was added to the mixtures and incubated overnight at 4°C. Nanoparticles were dialyzed against phosphate-buffered saline (PBS) and used for further study. Approximately 0.25 mg of protein was conjugated to each 1 mg of NP-GaN:Eu³⁺ and approximately 10 mg of protein was conjugated to 1 ml of 50 % (v/v) fluorescein-NPs suspension (fluoro-NPs). Specific lectins used in this study were provided by Dr. V. Antonyuk (LectinoTest Laboratory, Lviv, Ukraine).

Human leukemia Jurkat T-cells were grown in RPMI-1640 medium, while murine leukemia L1210 cells were grown in the DMEM medium, both supplemented with 10 % heat-inactivated fetal calf serum and gentamycin (50 mg/ml), both from Sigma Chem. Co., USA. The cells were cultured in a humidified atmosphere at 37°C and 5 % CO₂. Apoptosis in cell population was induced by etoposide (1 μg/ml) for 24 hours, and cell necrosis was induced by 50 μM of propidium iodine (1 μg/ml of cell suspension) to visualize dying cells; cells were also counterstained with 1:100,000 (w/v) acridine orange solution to visualize viable cells. In the experiment for visualization of NP-GaN:Eu³⁺ binding nanoparticles were visualized by fluorescent mode, while cells were visualized by DIC mode. ImageJ software was used for image processing, deconvolution and generation of color composite pictures. Deconvolution of image data (where mentioned) was carried out with the plugins Diffraction PSF 3D and Iterative Deconvolve 3D, freely available at http://www.optimas.com/imagej.html.

Photoluminescence (PL) of NP-GaN:Eu³⁺ samples was excited by the 300 nm cw-Ar laser (INNOVA 200 of Coherent) and 532 nm He-Ne laser and PL spectrum was analyzed by spectrometer (HR4000 Ocean Optics). PL spectrum of fluoro-NPs was analyzed using 425 nm excitation wavelength. The structural properties of the NP-GaN:Eu³⁺ powders were studied by transmission electron microscopy (TEM).

Based on our previous XRD results [9] it has been found that the NP-GaN:1.5 %Eu³⁺ characterize by ~30 nm size and hexagonal shape. Moreover, from Fig. 1A it can be seen that the grains have tendency to agglomeration creating bigger grains of order of ~150–200 nm.

Using fluorescent microscopy Fluoro-NPs were characterized as a heterogeneous population of particles with the size range of 0.5–10 μm. From this population by means of differential centrifugation we isolated a fraction of NPs with the size range 500–1000 nm (Fig. 1B), which constituted 27,31 % of general population if judged by measured particles absorbance using NanoDrop spectrophotometer.

In our recent work [10] it has been shown that there are different excitation channels for the europium ions doped into GaN nanoparticles: indirect (excitation below ~360 nm) and direct ones. As it can be seen from Fig. 2, at direct excitation, nanoparticles are characterized by very narrow (~10 nm) red emission line at ~614 nm related to 5D4 → 7F2 transition of Eu³⁺ ion. This narrow emission band makes this material very promising for precise identification of nanoparticles in multicolored detection systems. Thus, this emission band has been used as a signature of NP-GaN:Eu³⁺ when using the green excitation wavelength (532 nm). Nevertheless, the inset in Fig. 2, shows also the emission spectrum obtained for NP-GaN:Eu³⁺ at indirect excitation. In this case, the europium related signal is much weaker and less clear spectrally. However, strong and wide emission band appears...
between 500 and 800 nm together with the emission band centred at 360 nm. These bands are related to surface/defect states and NP core of GaN nanoparticles, respectively [11, 12]. Thus, this gives us also a new possibility of changing the detection signal depending on the excitation wavelength from spectrally narrow in red to broad but very intense in green.

Emission spectra of fluoro-NPs do not differ significantly from that of fluorescein itself with emission maximum at 514 nm, thus the created nanoparticles can be easily used in all devices [13], equipped with filters aimed to detect fluorescein or its conjugates (Fig. 2). The full width at half maximum of fluorescein fluorescent spectra equals to ~40 nm.

A set of lectins was screened for their ability to bind intact, apoptotic or necrotic cells [4] and unpublished data. Fluorescent or luminescent nanoparticles were synthesized and conjugated with appropriate lectin, as described in the experimental part. We used α-D-mannose specific lectin from spring snowflake *Leucojum vernum* (LVA) for the detection of apoptotic cells and branched N-glycan specific lectin - wheat germ agglutinin (WGA) for detection of necrotic cells. These lectins were previously chosen as those possessing a high activity towards dying cells (apoptotic and necrotic, correspondingly). Nanoparticles conjugated with bovine serum albumin (BSA) served as control.

Incubation of LVA lectin-conjugated NP-GaN:Eu³⁺ with a population of intact (Fig. 3 A,B) and apoptotic (Fig. 3 E,F) human leukemia Jurkat T-cells revealed their preferential binding to apoptotic cells. Nanoparticles were localized on the surface of cells and thus the integrity of the cell was not violated. The same NP-GaN:Eu³⁺ proved to be effective in providing both green (Fig. 3 A,E) and red (Fig. 3 B,F) fluorescence by simple changing the excitation wavelength. WGA-conjugated fluoro-NPs were preferentially bound by the necrotic murine leukemia L1210 cells (Fig. 3 G), and only insignificant binding by the intact cells of this line was observed (Fig. 3 C). These nanoparticles were also bound with the surface of necrotic cells without violating cell integrity (controlled by DIC and phase microscopy). Fluoro-NPs conjugated with BSA (serving as control for binding specificity) did not bound both intact or necrotic cells (Fig. 3 D, H). Fluoro-NPs were successfully observed in the green channel using a filter set suited for fluorescein isothiocyanate (FITC) conjugates, and thus can be detected in most of fluorescent devices.

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**Fig. 2.** Photoluminescence spectra of GaN:Eu³⁺ nanoparticles, excitation at 300 nm and 532 nm, compared to reference spectra of fluorescein conjugate (taken from Carl Zeiss web-site), with excitation at 490 nm. Fluorescein was incorporated into the core of fluoro-NP used in our study. The full width at half maximum for fluorescein equals to 40 (540–500) nm while for GaN:Eu³⁺ nanoparticles it equals to 10 (618–608) nm. The insert at the top indicate the size of corresponding particles.

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**Fig. 3.** Binding of lectin-conjugated luminescent and fluorescent nanoparticles by intact (upper row) and dying – apoptotic (D,E) and necrotic (F,G) leukemia cells (lower row). Please note, that nanoparticles signicantly stronger bind dying cells then intact one. A, B, D, E – represents binding of LVA-conjugated NP-GaN:Eu³⁺. C, F – represents binding of WGA-conjugated fluoro-NPs. D, H – represents non binding of BSA-conjugated fluoro-NPs (control nanoparticles).

In C and D living cells are counterstained with 1:100,000 (w/v) acridine orange solution (fain green), in G and H dead cells are counterstained with propidium iodine (1 μg/ml) solution to visualize nuclei of dead cells (red). White bar correspond to 5 μm.

microscope or flow cytometers [13]. They provided a vivid signal due to the high fluorescein content in the nanoparticles core; at the same time inert polymeric core isolated fluorescein molecules from interaction with the environment, preventing oxidation and other unwanted interactions.

Thus we described the efficient use of two different types of bioconjugated nanoparticles, targeted to detect dying apoptotic and necrotic cells. Lectin molecules, conjugated to these nanoparticles can be substituted by other lectins and/or antibodies, recognizing other specific cellular markers. Studied nanoparticles provided different size range as well as spectral characteristics: ~35 nm for NP-GaN:Eu³⁺ with narrow (~10 nm) red emission at 614 nm and bigger fluoro-NPs of 500–1000 nm with broader (~40 nm) green emission at 514 nm. Fluorescein and its derivatives are widely used in fluorescent applications, while incorporation of fluorescein into polystyrene nanoparticles allowed us to achieve much higher concentration of the dye (0.5 %), and as a result, much intense signal compared to fluorescein isothiocyanate-labeled proteins alone. The narrow emission band of NP-GaN:Eu³⁺ makes them ideal for use in multicolor systems for biodetection, especially for in vivo detection as living cells and tissues possess higher transparency of in the red spectral range.

4 Conclusions

We demonstrated an effective utilization of LVA lectin-conjugated luminescent GaN:Eu³⁺ nanoparticles and WGA lectin-conjugated fluorescent fluorescein-containing polystyrene nanoparticles for the detection of dying cells (apoptotic and necrotic correspondingly). Both types of nanoparticles provided high specificity, bright fluorescence and low photobleaching, and they can be easily used in most types of fluorescent microscopy and flow cytometry. Besides, GaN:Eu³⁺ nanoparticles emit fluorescence in a very narrow red spectral range, making them a very useful fluorescent nanolabel for multicolor in vivo detection systems.

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6 References


7 Abbreviations

NP – nanoparticle
fluoro-NP – fluorescein-containing polystyrene nanoparticles
NP-GaN:Eu³⁺ – Gallium nitride nanoparticles doped by europium ions

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