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BRIEF METHOD

Highly Stable Fluorescent Nanocrystals as a Novel Class of Labels for Immunohistochemical Analysis of Paraffin-Embedded Tissue Sections

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The recent development of nanotechnology has potentiated the appearance of a new class of highly fluorescent and homogeneous semiconductor nanocrystals (NCs) in the 2- to 6-nm size range termed "quantum dots" (Bruchez et al, 1998; Chan et al, 1998). These NCs can be excited at any wavelength yet will emit with the same characteristic fluorescence spectrum regardless of the excitation wavelength. Varying the material used for NCs and their size, one can obtain a spectral range of emission from 400 nm to 2 μm. Emission wavelengths of CdSe NCs passivated with a ZnS layer (CdSe/ZnS NCs) can be tuned from the blue to the red regions by changing the particle size, for example (Bruchez et al, 1998). Therefore, NCs of various sizes may be excited with light of a single wavelength, resulting in many emission colors that may be detected simultaneously. In comparison to organic dyes, NCs show similar or slightly lower quantum yields but demonstrate much larger absorption cross-sections and impressively reduced photobleaching rates. NC emission was found to be 100-fold more resistant to photobleaching than the fluorescent dye rhodamine 6G (Chan and Nie, 1998).

To date, NCs that are homogeneous in size (5% variation) have been solubilized in a water by methods of surface chemistry and conjugated with some proteins or DNA molecules (for review, see Chan et al, 2002). Despite the breakthrough advances in applications of the fluorescent NCs and NC-based bioconjugates for multiplexed optical encoding (Han et al, 2001), in in vitro immunoassays (Chan and Nie, 1998) and in DNA hybridization (Pathak et al, 2001), no data demonstrating their applicability in immunohistochemical analysis have been published yet. This report describes the first application of NC antibody (NC-Ab) conjugates in analysis of formaldehyde-fixed paraffin-embedded tissue sections.

NCs with CdSe core and ZnS surface shell were synthesized according to protocol adapted from Hines and Guyot-Sionnest (1996). Briefly, an appropriate amount of trioctylphosphine oxide (Aldrich Chemical, Milwaukee, Wisconsin) was heated to 180° C under argon, dried, and degassed at this temperature under vacuum. Then, trioctylphosphine oxide was heated to 340° C under argon flow and intensive stirring. Solutions of dimethylcadmium (Strem, Bischheim, France) and selenium (elemental powder; Aldrich) precursors in trioctylphosphine (Fluka, Buchs, Switzerland) were injected through syringe in less than 1 second. The reaction was cooled to 300° C and a 1 M solution of dimethylzinc (Aldrich) in heptane and a solution of hexamethyldisilthiane (Fluka) were added dropwise under vigorous stirring. The reaction mixture was cooled to 50° C, and NCs were precipitated out from the solution by adding anhydrous methanol followed by centrifugation at 14,000 rpm. The precipitate was washed with methanol, and NCs were dissolved in chloroform (Sigma Chemical, St. Louis, Missouri). The procedure that we describe permits the synthesis of homogeneous NCs emitting the fluorescence from nearly 500 nm to 620 nm depending on their diameters. For this article we have used perfectly homogeneous NCs (Fig. 1A) possessing a CdSe core of approximately 3.6 nm in diameter with an epitaxially grown ZnS shell of several monolayers in thickness.

NCs were solubilized in the aqueous solution by treating their surface with cysteine. Briefly, 200 μl of 10 mg/ml solution of DL-Cys (Aldrich) in methanol was

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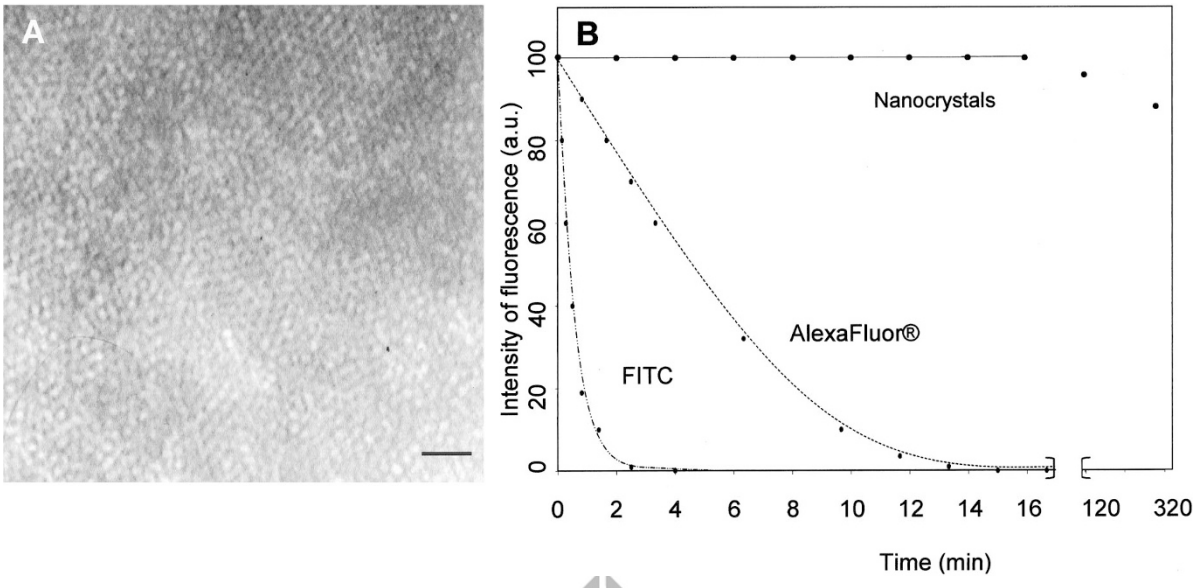

Figure 1.
Electron microscopy and photostability of CdSe/ZnS nanocrystals. A, Electron microscopy photographs were recorded with the electron microscope (model JEM-100CX, with screen magnification 100,000; JEOL, Tokyo, Japan). Bar = 20 nm. B, Time-dependent photobleaching curves for CdSe/ZnS nanocrystals and the dyes Alexa Fluor488 and FITC recorded with the fluorescence microscope Nikon Diaphot 300. Samples of dilute nanocrystals or dyes were deposited and spread on glass coverslips. Continuous-wave excitation was provided by a Nikon super high-pressure mercury lamp HB10101AF (Nikon Company, Tokyo, Japan).

added to 1 ml of 20 mg/ml solution of NCs in chloroform until the solution became cloudy. Then NCs were precipitated out by centrifugation at 14,000 rpm, washed three times with methanol to ensure removal of nonreacted Cys, dried under vacuum, and dissolved in a water by adding dropwise a 1 M solution of NaOH (Sigma) followed by sonication. The sample was recentrifuged at 14,000 rpm, and the pellet was redissolved in 1 ml of 0.1 M sodium phosphate, pH 7.3. The resulting water-soluble NCs possess bright orange photoluminescence with the emission maximum at 590 nm and quantum yield exceeding 50% at room temperature. Figure 1B shows excellent photostability of water-soluble nanocrystals compared with the FITC and AlexaFluor 488 (Interchim, Montluçon, France). The nanocrystal's emission in described experimental conditions (time constant $t_{1/2}$ = 27 hours) was found to be nearly 380 times as stable as AlexaFluor 488 ($t_{1/2}$ = 4.3 minutes) and nearly 3800 times as stable as FITC ($t_{1/2}$ = 0.43 minutes) against photobleaching.

NCs were further conjugated with anti-mouse polyvalent immunoglobulins (Sigma) using carbodiimide chemistry where the EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; Sigma) was used as a cross-linker. The 500 μl of 20 mg/ml IgG solution in 0.1 M sodium phosphate (pH 7.3) was mixed with the 400 μl of 20 mg/ml NC solution in the same buffer to obtain a molar ratio of 1:1. Then a 100-μl aliquot of fresh 6.4 mg/ml EDC stock solution in water was added to the mixture. After incubation for 2 hours at room temperature, the sample was dialyzed against PBS, pH 7.2. The activity of prepared NC-Ab conjugate was tested in the dot-blot assay, in which it demonstrated conservation of Ab-antigen binding affinity, being able to detect 1 ng of antigen preincubated with the corresponding primary Abs.

We have further used active NC-Abs for immunohistochemical analysis of samples of skin basal cell carcinoma and tonsil tissues obtained from resected specimens submitted to the Department of Pathology, Hospital Robert Debré, Reims, France, for diagnosis. The tissues were fixed in 10% (v/v) phosphate-buffered formaldehyde solution at 4° C for 24 hours and embedded in paraffin. Sections were microdissected in 3-μm slices and dried at 56° C for 1 minute. The samples were deparaffinized for a total of 15 minutes in 1 ml of xylene and rehydrated by successive treatment with 100%, 95%, and 70% ethanol and with nuclease-free water; antigens were retrieved using microwave antigen retrieval (Katoh et al, 1997). Slides with skin basal cell carcinoma tissue sections were incubated with anticytokeratin (ImmunoTech, Marseille, France; clone KL1, dilution 1:50) primary Abs, and slides with tonsil tissue sections were stained with antileukocyte common antigen Abs (Dako, Glostrup, Denmark; clones 2B11+PD7/26, isotype IgG1 Kappa, dilution 1:50). Samples were then incubated with NC-Abs used as the secondary antibodies.

Both samples studied here demonstrated applicability of NC-Abs for highly specific analysis of corresponding antigens—cytokeratin in skin basal carcinoma (and adjacent normal skin) and leukocytes in tonsil tissue. Figure 2 shows results of immunohistological detection of leukocytes in tonsil tissue presented as an example. Panel B demonstrates specific labeling of the germinal center of lymphoid tonsil tissue with additional labeling of some small lymphocytes around the center. At the bigger magnifications (panels C and D) one can see clear positive signals issuing from the cellular membranes. It should be noted that neither nuclei nor background signals

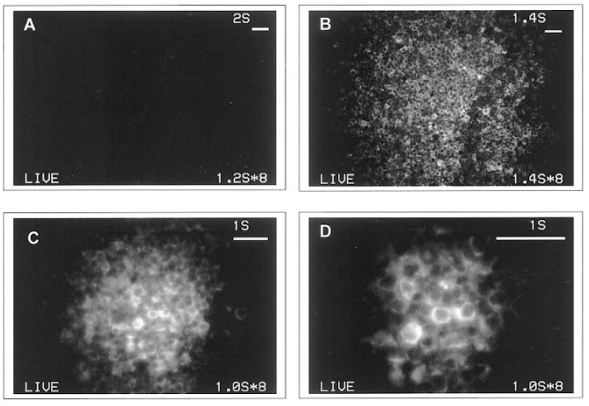

Figure 2.

Fluorescence images of tonsil tissue. B, C, and D, Samples were incubated with the antileukocyte common antigen (LCA) primary antibodies and stained with anti-mouse polyclonal immunoglobulins linked to fluorescent CdSe/ZnS nanocrystals. Panels B, C, and D represent fluorescence images of the same sample recorded at the ×10, ×20, and ×40 magnifications, respectively. A represents control: fluorescence image of the same sample incubated directly with the anti-mouse polyclonal immunoglobulins linked to fluorescent CdSe/ZnS nanocrystals without preincubation with the primary antibody. Images are recorded using a Nikon Diaphot 300 fluorescence microscope equipped with the filter combination for fluorescence excitation at 480 nm and detection of emission at 590 nm. Scale bars = 50 μm.

where detected in these experiments showing excellent specificity of lymphocyte membrane labeling. Immunolabeling of the cytokeratin in skin basal carcinoma (image not shown) also demonstrated excellent specificity in which neither nuclei labeling nor background nonspecific fluorescent signal in the absence of primary Abs was detected.

This study shows the availability and usefulness of NC-Ab conjugates for analysis of routine formalin-fixed, paraffin-embedded pathologic specimens. We are actually synthesizing homogeneous NCs of six different diameters (emitting six different colors), which may be conjugated with the primary Abs using the methods similar to those used in this article. The unique property of prepared NCs of various sizes that may be excited with a single wavelength, resulting in many emission colors that may be detected simultaneously, remains to be exploited in (at least) double-immunofluorescence techniques. These techniques strongly suffer from quenching of the fluorescence signal at excitation and its fading upon storage of specimens (Brandtzaeg et al, 1997). Impressive photostability of NCs should help to overcome these problems. The double-immunofluorescence technique applied to the analysis of pathologic specimens will be a subject of our next article.

References

Brandtzaeg P, Halstensen TS, Huitfeldt HS, and Valnes KN (1997). Immunofluorescence and immunoenzyme histochemistry. In: Johnstone AP and Turner MW, editors. Immunochemistry 2: A practical approach. Oxford: Oxford University Press, 71–130.

Bruchez M Jr, Moronne M, Gin P, Weiss S, and Alivisatos AP (1998). Semiconductor nanocrystals as fluorescent biological labels. Science 281:2013–2015.

Chan WCW, Maxwell DJ, Gao X, Bailey RE, Han M, and Nie S (2002). Luminescent quantum dots for multiplexed biological detection and imaging. Curr Opin Biotechnol 13:40–46.

Chan WCW and Nie SM (1998). Quantum dot bioconjugates for ultrasensitive nonisotopic detection. Science 281:2016–2018.

Han MY, Gao X, Su JZ, and Nie SM (2001). Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. Nat Biotechnol 19:631–635.

Hines MA and Guyot-Sionnest P (1996). Synthesis and characterization of strongly luminescent ZnS-capped CdSe nanocrystals. J Physiol Chem 100:468–471.

Katoh AK, Stemmler N, Specht S, and D'Amico F (1997). Immunoperoxidase staining for estrogen and progesterone receptors in archival formalin fixed, paraffin embedded breast carcinomas after microwave antigen retrieval. Biotech Histochem 72:291–298.

Pathak S, Choi SK, Arnheim N, and Thompson ME (2001). Hydroxylated quantum dots as luminescent probes for in situ hybridization. J Am Chem Soc 123:4103–4104.