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Peptide-Conjugated Quantum Dots

Imaging the Angiotensin Type 1 Receptor in Living Cells

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Summary

Peptide–quantum dot conjugates have been prepared by attaching angiotensin II (Ang II) to cadmium selenide/zinc sulfide core-shell nanocrystals using an 1-[3-(Dimethyamino)propyl]-3-ethylcarbo diimide hydrochloride (EDC) coupling. These conjugates have been used to image angiotensin I–expressing Chinese hamster ovary (CHO) cells in vitro. When CHO cells were incubated with Ang II before incubating with Ang II-conjugated quantum dots, we were able to block the binding of the dots. The Ang II–quantum dot conjugates did not bind to parental cells and showed similar staining patterns when compared with a commercially available Ang II Alexa 488™ conjugate.

Key Words

Nanocrystals; quantum dot; angiotensin II; peptide; fluorescence; Chinese hamster ovary cells; Alexa 488™.

1. Introduction

Cadmium selenide/zinc sulfide core-shell nanocrystals (quantum dots) are composed of a crystalline cadmium selenide core (2–7 nm in diameter) surrounded by a zinc sulfide shell (approx 1.5 nm thick). The small size of the core gives rise to unique properties that are not observed in bulk material. The fluorescent properties of quantum dots have potential applications in biological imaging and drug development. When compared to conventional dyes, it has been shown that quantum dots have several unique advantages, including increased quantum yields; increased photostability; narrower, Gaussian-shaped emission bands; and a continuous absorption above the first excitation feature

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(1–5). The fluorescence emission of quantum dots is size tunable: small quantum dots give rise to fluorescence toward the blue end of the spectrum and large quantum dots give rise to a fluorescence emission towards the red end of the spectrum ***(6–8)***.

Several groups have reported conjugating antibodies, proteins, peptides, and nucleic acids to quantum dots ***(9–18)***. In our laboratory, we have synthesized conjugates composed of small molecules and short peptides attached to quantum dots via either electrostatic interactions, acid base interactions, or direct covalent attachment ***(19–21)***. Our interests include the G protein-coupled receptors. This is a very broad family of receptors that regulates a wide range of physiological functions. Their ligands may be small molecules such as serotonin and dopamine or peptides such as angiotensin II (Ang II) and somatostatin. Because of the wide variety and different functions of peptide hormones, we have developed a methodology for attaching peptides to quantum dots. In this chapter, we describe how angiotensin II may be covalently attached to the surface of polymer-coated (AMP™) quantum dots. These peptide-nanoconjugates have been used to successfully image live Chinese hamster ovary (CHO) cells expressing the angiotensin I receptor.

2. Materials

1. AMP-coated cadmium selenide/zinc sulfide core-shell nanocrystals (Quantum Dot, Haywood, CA).
2. Borate buffer, 0.5 *M*, pH 8.5, 5X concentrate (Polysciences, Warrington, PA).
3. mPEG-Amine, mol wt = 5000, chromatographically pure (Shearwater, Huntsville, AL).
4. *N*-Hydroxysuccinimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (Aldrich, St. Louis, MO).
5. Sephadex PD-10 columns Sephadex TM G25-M (Pharmacia, Uppsala, Sweden).
6. CHO cells (Dr. Ted Ingami, Department of Biochemistry, Vanderbilt University, TN).
7. Human ang II (Sigma-Aldrich, St. Louis, MO).
8. Trypsin (Sigma-Aldrich).
9. Ang II, Alexa Fluor® 488 conjugate (Molecular Probes, Eugene, OR).
10. Phosphate-buffered saline (PBS): (1X solution): 0.1 *M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.5 m*M* Na_2HPO_4, 1 m*M* $MgCl_2$, 0.1 m*M* $CaCl_2$; pH to 7.4.
11. Inverted fluorescent microscope.
12. HBO 100 mercury lamp (Zeiss, Oberkochen, Germany).
13. Charge-coupled device (CCD) digital video camera (Roper, Tucson, AZ).
14. Glass-bottomed microwell dishes (MatTek, Ashland, MA).
15. Hemacytometer (Improved Neubauer) (BD Biosciences, Bedford, MA).
16. Swinging-bucket centrifuge.
17. Laminin-coated cover slips (BD Biosciences).
18. CO_2-controlled incubator.

19. Inverted light microscope.
20. Glutamine (Sigma-Aldrich).
21. Nonenzymatic dissociation solution (Sigma-Aldrich).
22. Glycine (Sigma-Aldrich).
23. Poly-D-lysine (Sigma-Aldrich).
24. Dialyzed fetal bovine serum (Sigma-Aldrich).
25. Penicillin-streptomycin (Invitrogen, Carlsbad, CA).
26. Dulbecco's modified Eagle's medium F12 Nutrient Mixture (HAM) (Gibco-BRL, Rockville, MD).
27. Angle-neck flasks (40 mL) (Nalgen Nunc, Rochester, NY).
28. Excitation/emission filter set: 485/20 and 515–530; 546/12 and 580–630.
29. KRH buffer: 60 m*M* NaCl, 2 m*M* KCl, 0.6 m*M* KH_2PO_4, and 4 m*M* HEPES.
30. Rotating shaker (Stoval, Greensboro, NC).
31. Polypropylene conical tubes (50 mL) (Becton Dickinson, Franklin Lakes, NJ).

3. Methods

The methods described next outline (1) the synthesis of Ang II, mPEG-amine-conjugated AMP-coated water cadmium selenide/zinc sulfide core-shell nanocrystals; (2) fluorescence imaging studies of CHO cells using the Ang II conjugates; and (3) fluorescence imaging studies of CHO cells using an Ang II Alexa Fluor 488 conjugate.

3.1. Synthesis of Ang II, mPEG-Amine-Conjugated AMP-Coated Cadmium Selenide/Zinc Sulfide Core-Shell Nanocrystals

We prepared nanoconjugates of Ang II as follows: A total of 0.3 mL of a 2 μ*M* solution of AMP-coated quantum dots in borate buffer at pH 8.5 was placed in a vial. These dots had a maximum emission at 585 nm and a full width at half maximum emission (FWHM) of 22 nm. Their quantum yield was 37% and they were a gift from Quantum Dot (batch no. 228-35). Then *N*-hydroxysuccinimide (35 μg, 30 nmol) dissolved in 0.1 mL of borate buffer and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (5.8 μg, 30 nmol) dissolved in borate buffer (0.1 mL) were each added, and the solution was stirred for 5 min at room temperature. mPEG-amine 5000 (0.6 mg, 12 nmol) and human Ang II (70 μg, 6 nmol) dissolved in borate buffer (0.1 mL) were next added, and the mixture was stirred at room temperature for 18 h. The conjugates were then purified using a Sephadex P-10 column eluted with borate buffer at pH 8.5. Fractions containing 0.1 mL of solution were collected from the column, and the product was shown to be in fractions three to nine, because these samples fluoresced brightly under a mercury lamp. Fractions four to eight were combined, and the concentration of the resulting nanoconjugate was determined using UV-vis spectroscopy and was determined using an extinction coefficient of 650,000 (Quantum Dot, personal

communication, September 2000). The conjugated dots were used to image cells with no further purification.

3.2. CHO Cells and Cell Cultures

CHO cells, a parental line and a stable line expressing the Ang II receptor type 1 (AT1) receptor, were a gift from Dr. Tad Inagami (Vanderbilt University, Nashville, TN). Cells were delivered frozen on dry ice in cryogenic vials at a concentration of 1×10^8/mL. On arrival, the vials were stored immediately in liquid nitrogen for later use. At the time of use, the vials were removed from the liquid nitrogen and freeze-thawed on ice. While thawing, the flasks were labeled as either CHO/parental or CHO/AT1. Cell medium, previously stored at 4°C, was removed and placed in a 37°C water bath until needed.

In the remainder of this section, each step was performed using an aseptic technique inside a laminar flow hood (*see* **Note 1**). Ten milliliters of medium was transferred by pipet into each labeled flask. Thawed vials were then sterilized by swabbing with alcohol. The vials were slowly opened, and the medium with the cells was transferred by pipet into the appropriately labeled flask. The flasks were next placed in a 37°C incubator at 5% CO_2, and the cells were allowed to grow until confluent.

Once confluent, the medium was aspirated off, the cells were rinsed with PBS, and then 3 mL of 0.1% trypsin was added. After approx 1 to 2 min, or when the cells began to round up and detach, 3 mL of medium was added to disperse the cells and to inhibit the trypsin (*see* **Note 2**). Cells from each flask were then transferred to an appropriately labeled 50-mL conical tube and centrifuged at 500*g* for 5 min. The supernatant was aspirated off, and the cells were resuspended in fresh medium. The cells were next counted with a hemacytometer and their concentration was determined (*see* **Note 3**). The cell solution was diluted to 1×10^5 cells/mL, and 150 µL was added to the glass cover slips of poly-D-lysine-coated sterile MatTek microwell dishes. Cells were then incubated at 37°C with 5% CO_2 for 3 h to allow the cells to attach. Once the cells had attached, 2 mL of additional medium was added to each of the dishes. The dishes were then returned to the incubator and allowed to grow for 3 d or until 50% confluent.

3.2.1. Detection Using Ang II-Conjugated Quantum Dots

Ang II AT1 receptors expressed on the surface of CHO cells can be detected directly by using Ang II-conjugated quantum dots and Ang II-conjugated Alexa 488 as a control. **Subheadings 3.2.1.1.** and **3.2.1.2.** describe procedures that were developed for the direct detection of the AT1 receptor on the surface of living CHO cells with angII–quantum dots and Ang II–Alexa 488, respectively.

3.2.1.1. Ang II–Quantum Dot

1. Aspirate off the excess KRH buffer on the cells covering the cover slip glass of the microwell dish, being careful to avoid removal of attached cells.
2. Add 50 µL of Ang II–quantum dots (200 n*M*) in borate buffer (pH 8.4) to the cover slip glass of the microwell dish.
3. Incubate the dish at room temperature for 5 min with no agitation (*see* **Note 4**).
4. Quickly rinse the dish three times with KRH.
5. Add 100 µL of KRH to the cells covering the cover slip glass well of the microwell dish.
6. Immediately image the Ang II–quantum dot-labeled cells.

3.2.1.2. Ang II–Fluorophore (Alexa 488)

1. Carefully aspirate off the excess KRH buffer from the cells covering the cover slip glass of the microwell dish to avoid removal of attached cells.
2. Add 50 µL of Ang II–Alexa 488 (2.5 µg/mL) in KRH buffer (pH 7.4) to the cover slip glass of the microwell dish.
3. Incubate the dish at room temperature for 5 min with no agitation.
4. Quickly rinse the dish three times with KRH.
5. Add 100 µL of KRH to the cells covering the cover slip glass well of the microwell dish.
6. Immediately image the Ang II–Alexa 488-labeled cells.

3.3. Fluorescent Imaging

To image cells fluorescently on cover glass microwell dishes labeled directly with Ang II–quantum dots or Ang II–Alexa 488 after incubation with SA-quantum dots or SA–Alexa 488 requires an inverted fluorescent microscope equipped with a transmitted-light illumination system for the initial bright-field examination, an incident-light illuminator for the excitation of fluorophores, and a filter cube with the appropriate filter set. For convenience, the scope should contain a phototube for mounting either a conventional 35-mm or an electronic CCD digital video camera. If a digital video camera is used, it must be interfaced with a computer. The steps described for imaging Ang II–quantum dot- and Ang II–Alexa 488-labeled cells in this chapter use an Axiovert 110 (Zeizz) equipped with an HBO 100 mercury lamp (Zeiss) and a Photometric CoolSnap™ digital CCD camera (Roper). However, these steps can be repeated using a comparable imaging system.

3.3.1. Imaging of CHO Cells

1. Position cells on the cover glass of microwell dishes labeled with either Ang II–quantum dots or Ang II–Alexa 488 on the stage of an inverted fluorescent microscope.

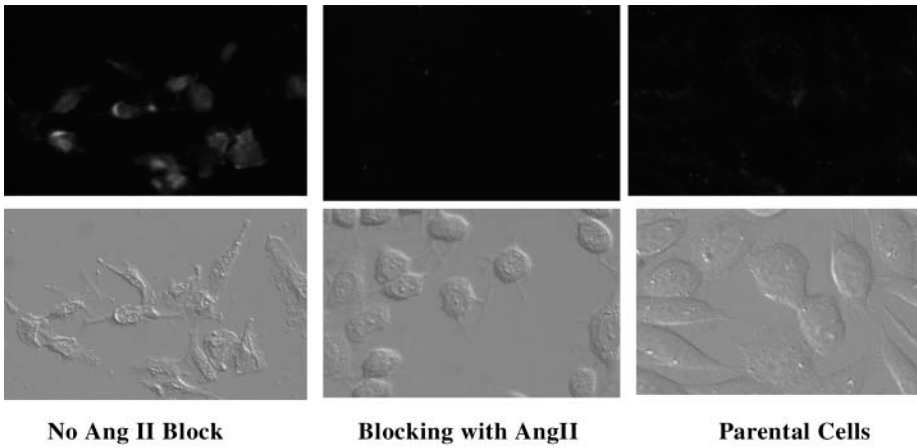

Fig. 1. Imaging CHO cells with Ang II–quantum dot conjugates. The upper panels show the fluorescence images, and the lower panels show the bright-field images. Cells are clearly visible in all of the bright-field images; however, only the cells incubated with Ang II conjugates that express the AT1 receptor are seen to be fluorescent (top left panel). When these cells were preincubated with a 100 μ*M* solution of Ang II, the binding of Ang II-conjugated quantum dots was blocked (top center panel). Finally, when parental cells that did not express the receptor were incubated with the conjugate, no fluorescence was observed (top right panel).

2. Using transmitted light, bring the cells on the cover glass into focus and capture bright-field image using a digital CCD camera.
3. Using 488 nm of incident light, excite the cells labeled by Ang II–quantum dots or Ang II–Alexa 488, and obscure the fluorescence through a 485/20 and 515–530 band-pass filter for Ang II–Alexa 488 and through a 546/12 and 580–630 filter for Ang II–quantum dots.
4. To capture an image, illuminate the Ang II–quantum dot-labeled cells on the cover glass of the dishes with incident light for a minimum of 2 min to induce maximal fluorescence (*see* **Note 5**). Immediately capture images of Ang II–Alexa 488–labeled dishes to avoid bleaching.
5. To ensure that Ang II–quantum dots used to label cells have not aggregated while labeling, examine imaging fields for Ang II–quantum dot clusters. When aggregates or clusters are present, it is assumed that Ang II–quantum dots were not in the solution while labeling and that Ang II–quantum dot labeling was compromised (*see* **Note 6**).

In our initial experiments, we were able to label CHO cells with fluorescent cadmium selenide core-shell nanocrystals that were conjugated to Ang II. We showed that this labeling was specific for cells expressing the AT1 receptor and that there was no labeling of parental cells that did not express the AT1 receptor (**Fig. 1**). Furthermore, the specific binding of Ang II-conjugated

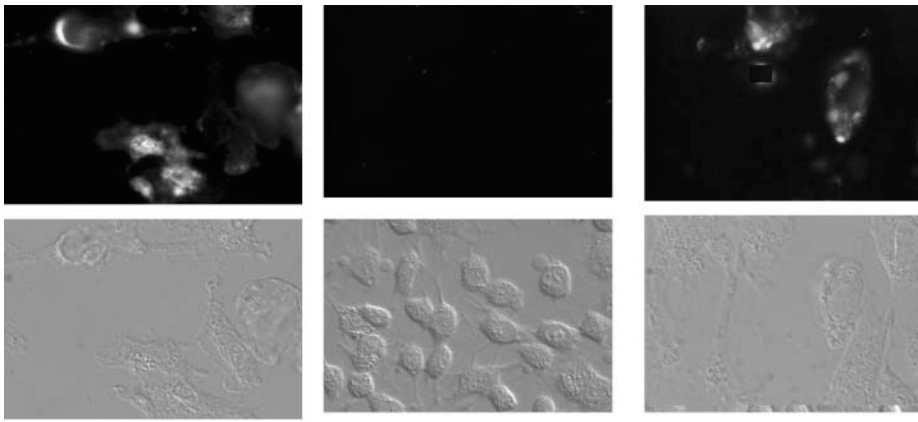

Fig. 2. Imaging CHO cells expressing AT1 receptor using Ang II–quantum dot conjugates and Ang II–Alexa 488. The upper panels show the fluorescent images, and the lower panels show the bright-field images. Cells are clearly visible in all of the bright field images. Cells expressing the AT1 receptor specifically bind Ang II–quantum dot conjugates (top left panel). Similar binding was observed when the cells were incubated with Ang II–Alexa 488 (top right panel). This specific binding of Ang II–quantum dot conjugates was blocked by preincubation of AT1-expressing cells with a 100-μ*M* solution of Ang II (top center panel).

nanocrystals to cells expressing the AT1 receptor was blocked by preincubation of cells with excess Ang II (**Fig. 1**).

The results of our initial experiment demonstrate that the Ang II-conjugated quantum dots bind specifically to the CHO cells expressing the AT1 receptor. We then compared the specificity of the Ang II–conjugated quantum dots with the angiotensin dye conjugate (Ang II–Alexa 488). Cells were incubated with each conjugate and their fluorescence was compared. These images are shown in **Fig. 2**.

4. Notes

1. In spite of the introduction of antibiotics, contamination by microorganisms remains a major problem in tissue culture. Correct aseptic technique should provide a barrier between microorganisms in the environment outside the culture. The procedure used for equipping and practicing aseptic technique in a laboratory has been described in a number of sources ***(22,23)***. Inability to follow the steps in aseptic technique will likely result in bacterial contamination that may alter the biochemical behavior and cell-surface protein expression of the cell line. Taking time to obtain advice from personnel of other laboratories experienced in aseptic technique can greatly facilitate success in this area.

2. Although some cell lines can be dislodged from the bottom of the flask by gentle and repeated pipetting of the medium over the cells, trypsinizing the cells has the advantage of producing a single cell suspension. However, overtrypsinizing may reduce viability, and undertrypsinizing can result in cell clumps. Furthermore, cells treated with trypsin may require one or more days after plating before reexpression of surface proteins. If viability is a concern, a commercially available nonenzymatic dissociation buffer solution can be used (e.g., from Sigma-Aldrich). These dissociation solutions lack magnesium.
3. The concentration of a cell suspension may be determined by placing cells in an optically flat chamber, known as a hemacytometer, under a microscope. The cell number within a defined area of the hemacytometer can then be counted and the cell concentration derived from the count. Detailed instructions on how to use a hemacytometer (Improved Neubauer) are described elsewhere ***(24)***. An alternative approach to the hemocytometer is electronic particle counting. This method requires the use of an automated system called a Coulter Counter. In this system, cells in suspension are drawn through a fine orifice. As each cell passes through the orifice, it causes a change in current. This change produces a series of pulses, which are then counted ***(25)***.
4. Depending on the cell type, rapid agitation may cause cells to detach; therefore, we recommend gently rotating dishes manually. Because borate buffer is harsh on cells, we incubate cells for no more than 15 min to avoid dissociation. However, this will vary with the cell type and number of days that they are cultured. Cells tend to dissociate most readily if cultured less than 2 d and/or are overgrown.
5. When imaging quantum dots, it is necessary to illuminate for 2 to 3 min before maximum fluorescent intensity is reached. The rate of quantum dot bleaching is approx 2%/min when illuminated continuously after reaching maximum fluorescence intensity. This is in contrast to conventional fluorophores, which are typically bleached entirely within minutes after beginning to illuminate ***(26)***.
6. When imaging, it is important to recognize when aggregates have formed. Our experience is that aggregates occur unpredictably. Before adding quantum dots for labeling, we advise placing Ang II–quantum dots on a clean cover glass of a dish and imaging at ×40 to determine whether aggregates have already formed. This will confirm whether the Ang II–quantum dots should be used for labeling. If Ang II–quantum dots are not aggregated before adding but aggregate after incubation with cells, then the problem resides with the incubation step.

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