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Upconverting nanophosphors as reporters in a highly sensitive heterogeneous immunoassay for cardiac troponin I

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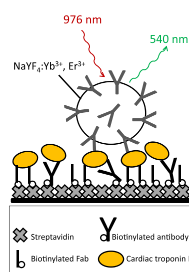
HIGHLIGHTS

- Detection of attomole analyte quantity in microwell using upconversion luminescence.
- Upconverting nanophosphors enable detection of femtomolar analyte concentration.
- Excellent analyte recovery from plasma in upconversion luminescence based immunoassay.

GRAPHICAL ABSTRACT



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ABSTRACT

Photon upconverting nanophosphors (UCNPs) have a unique capability to produce anti-Stokes emission at visible wavelengths via sequential multiphoton absorption upon infrared excitation. Since the anti-Stokes emission can be easily spectrally resolved from the Stokes' shifted autofluorescence, the upconversion luminescence (UCL) is a highly attractive reporter technology for optical biosensors and biomolecular binding assays — potentially enabling unprecedented sensitivity in separation-based solid-phase immunoassays.

UCL technology has not previously been applied in sensitive detection of cardiac troponin I (cTnI), which requires highly sensitive detection to enable accurate and timely diagnosis of myocardial infarction. We have developed an UCL-based immunoassay for cTnI using $NaYF_4$: Yb^{3+}, Er^{3+} UCNPs as reporters. Biotinylated anti-cTnI monoclonal antibody (Mab) and Fab fragment immobilized to streptavidin-coated wells were used to capture cTnI. Captured cTnI was detected from dry well surface after a 15 min incubation with poly(acrylic acid) coated UCNPs conjugated to second anti-cTnI Mab. UCL was measured with a dedicated UCL microplate reader.

The UCL-based immunoassay allowed sensitive detection of cTnI. The limit of detection was 3.14 ng L^{-1}. The calibration curve was linear up to cTnI concentration 50,000 ng L^{-1}. Plasma recoveries of added cTnI were 92–117%. Obtained cTnI concentrations from five normal plasma samples were 4.13–10.7 ng L^{-1} (median 5.06 ng L^{-1}). There is yet significant potential for even further improved limit of detection by reducing non-specifically bound fraction of the Mab-conjugated UCNPs. The assay

Abbreviations: BGG, bovine gamma globulin; BSA, bovine serum albumin; cTnI, cardiac troponin I; Fab, fragment antigen binding; EDC, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide; LoB, limit of blank; LoD, limit of detection; Mab, monoclonal antibody; PAA, poly(acrylic acid); SA, streptavidin; SD, standard deviation; sulfo-NHS, N-hydroxysulfosuccinimide; UCL, upconversion luminescence; UCNP, upconverting nanophosphor.

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background with zero calibrator was over 40-fold compared to the background obtained from wells where the reporter conjugate had been excluded.

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1. Introduction

Photon upconverting nanophosphors (UCNPs) are close to spherical, monodisperse nanoparticles consisting of an inorganic host lattice and lanthanide dopant ions [1]. They have the unique capability to convert infrared radiation into emission at visible wavelengths. The anti-Stokes emission is achieved by the sequential absorption and “piling up” of two or more infrared excitation photons in energy via equally spaced long-lived excited states of lanthanide dopants. Upconversion luminescence (UCL) enables practically background-free photoluminescence detection, since the anti-Stokes emission can be efficiently spectrally resolved from the Stokes' shifted autofluorescence, which occurs at the excitation wavelength or longer wavelengths [1,2]. Thus, UCL technology shares the autofluorescence elimination as a common advantage over conventional fluorescence detection with time-resolved fluorometry, where long-lived luminescence emission from lanthanide complexes is measured after a short delay from excitation, thus avoiding the short-lived autofluorescence [3]. UCL enables however more simple and affordable instrumentation since there is no need for pulsed excitation and temporal gating in luminescence detection and low-cost, compact solid-state infrared laser diodes can be used as excitation source.

Cardiac troponin (cTn) I and T are biochemical markers used in the diagnosis of myocardial infarction (MI). To improve diagnostic accuracy and to enable faster diagnosis of MI, the Global MI Task Force proposes that troponin assays should be able to measure cTn elevations above the 99th percentile concentration of a healthy population [4]. cTnI exists as ternary cTnI-cTnT-TnC (I-T-C) complex in heart tissue and is found still in complex with TnC in the blood of MI patients. The molecular weight of cTnI is 29 kDa [5,6]. Commercial high-sensitivity (hs) cTn immunoassays, Architect hs-cTnI (Abbott Diagnostics) and Elecsys hsTnT (Roche Diagnostics), are available for clinical use in a number of countries, with detection based on chemiluminescence [7] (Architect) or electrochemiluminescence (Elecsys) [8]. Three research assay platforms for the ultrasensitive detection of cTnI have been developed, with detection based on single molecule counting [9], on Verigene nucleic acid detection protocol [8] and on digital ELISA [10,11].

To best of our knowledge, there are no reports on UCL reporter technology being used for the sensitive detection of cTnI and our work presents the most sensitive heterogeneous immunoassay utilizing UCNPs as reporters published so far. Recently, *e.g.* Huang et al. have demonstrated detection beta-subunit of human chorionic gonadotropin (β-hCG) in heterogeneous immunoassay [12], Peng et al. have used UCL in homogenous sensing of Zn^{2+} from real biological samples [13], Hu et al. [14] have reported on a competitive immunoassay for the detection of fluoroquinolones in animal-derived foods, and Li et al. [15] reported on an immune sandwich assay for the detection of carcinoembryonic antigen based on the joint use of UCNPs and magnetic beads. Majority of the recent development on biosensing utilizing UCNPs has focused on rapid and easy-to-perform homogeneous assays based on upconversion resonance energy transfer [16,17], with assay sensitivities reaching nanomolar concentrations at best. However, for an analyte such as cTnI, assay sensitivities in the subpicomolar range are required. In our study, a highly sensitive heterogeneous immunoassay for cTnI using UCNPs as reporters was implemented to demonstrate the suitability of UCNPs as a feasible reporter alternative in detecting low levels of cTnI.

2. Materials and methods

2.1. Materials and reagents

Oleic acid capped Upcon™ UCNPs ($NaYF_4$:Yb^{3+}, Er^{3+}, particle dimensions 29 × 36 nm), streptavidin (SA) coated microtitration plates (SA96-C8 White), assay buffer (colorless) and wash concentrate were from Kaivogen (Turku, Finland). Poly(acrylic acid) (PAA, Mw 2000), bovine gamma globulin (BGG; G-5009) and D-trehalose were purchased from Sigma–Aldrich (St. Louis, MO), and N-hydroxysulfosuccinimide (sulfo-NHS) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) were purchased from Fluka (Buchs, Switzerland). Biotin isothiocyanate was synthesized at the Department of Biotechnology, University of Turku (Turku, Finland). Bovine serum albumin (BSA) was purchased from Bioreba (Nyon, Switzerland) and native mouse IgG from Meridian Life Science (Saco, ME). Denatured mouse IgG was prepared from native IgG by heat denaturation (+63 °C, 30 min). Casein and heparin were purchased from Calbiochem (LaJolla, CA) and non-fat milk powder from Valio (Helsinki, Finland). All other reagents used were of analytical grade. Two cTnI specific monoclonal antibodies (Mabs, clones 19C7 and 625) were purchased from HyTest (Turku, Finland). Recombinant fragment antigen binding (Fab) was cloned from the hybridoma cell line (clone 9707) of Medix Biochemica (Kauniainen, Finland) and produced and site-specifically biotinylated with EZ-Link® Maleimide-PEG_2-Biotin (Thermo Scientific, Thermo Fisher Scientific Inc., Waltham, MA) as described by Ylikotila et al. [18]. 9707-Fab was used instead of 9707-Mab, because eliminating the Fc-part from one of the capture antibodies has been shown to significantly reduce non-specific signal increase caused by sample matrix related interferences [19]. 19C7-Mab was biotinylated using 10x molar excess of biotin isothiocyanate as described earlier [20]. Human cardiac troponin I-T-C complex (native, human heart tissue derived) was purchased from HyTest.

2.2. UCNP surface chemistry and conjugation to Mab

UCNPs were coated with PAA to obtain a hydrophilic surface carrying high density of carboxyl groups and enabling covalent conjugation to 625-Mab using sulfo-NHS/EDC chemistry. PAA coated UCNPs have been shown to be water dispersible and to have excellent colloidal stability [21]. First, oleic acid was washed from the particle surface by using a modified method of Bogdan et al. [22]; 5 mg of UCNP in toluene was pelleted by centrifuging (Eppendorf Centrifuge 5418, Hamburg, Germany) at 16,873*g* for 30 min. The pellet was dispersed to 1 mL of 0.1 M HCl by ultrasonication (Finnsonic m03, Finnsonic Oy, Lahti, Finland). The reaction was incubated in rotation at ambient temperature for at least 5 h, after which the UCNPs were recovered by centrifuging as before and washed with 1 mL of mQ-H_2O. Next, 1 mL of 2.5 wt% PAA solution (pH adjusted to 9 with 5 M NaOH) was added on top of the UCNP pellet and the pellet was dispersed by ultrasonication (Finnsonic m03, 3 min), vigorous mixing in a vortex mixer and

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