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Comparison of infrared-excited up-converting phosphors and europium nanoparticles as labels in a two-site immunoassay

Telle Ukonaho *, Terhi Rantanen, Laura Jämsen, Katri Kuningas, Henna Päkkilä, Timo Lövgren, Tero Soukka

Department of Biotechnology, University of Turku, Tykistökatu 6A, FIN-20520 Turku, Finland

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Abstract

Research in the field of immunoassays and labels used in the detection has been recently focused on particulate reporters, which possess very high specific activity that excludes the label as a sensitivity limiting factor. However, the large size and shape of the particulate labels may produce additional problems to immunoassay performance. The aim of this work was to study with two identical non-competitive two-site immunoassays whether up-converting phosphor (UCP) particles are comparable in performance with europium(III) chelate-dyed nanoparticles as particulate labels. In addition we strived to verify the common assumption of the photostability of up-converting phosphor particles supporting their potential applicability in imaging. Detection limits in two-site immunoassay for free prostate-specific antigen (free-PSA) were 0.53 ng L^{-1} and 1.3 ng L^{-1} using two different up-converting phosphors and 0.16 ng L^{-1} using europium(III) nanoparticle. Large size distribution and non-specific binding of up-converting phosphor particles caused assay variation in low analyte concentrations and limited the analytical detection limit. The non-specific binding was the major factor limiting the analytical sensitivity of the immunoassay. The results suggests the need for nanoscaled and uniformely sized UCP-particles to increace the sensitivity and applicability of up-converting phosphor particles. Anti-Stokes photoluminescence of up-converting phosphor particles did not photobleach when measured repeatedly, on the contrary, the time-resolved fluorescence of europium nanoparticles photobleached relatively rapidly.

Keywords: Particulate label; Up-converting phosphors (UCPs); Europium nanoparticles; Photobleaching

1. Introduction

Different reporter molecules, such as radioisotopic, chemiluminescent, electroluminescent, enzymatic, colorimetric, and especially fluorescent labels, have been evaluated for their use in immunoassays since 1960s. Unfortunately, most of the detection methods based on conventional fluorescent labels are of limited sensitivity. Therefore, development has continued towards more sensitive fluorescence based detection methods, e.g. long lifetime lanthanide chelates and time-resolved detection, which enables efficient elimination of autofluorescence [1,2].

In recent years, research has been focused on particulate reporters [3,4]. These labels possess very high specific activity that excludes the signal as a sensitivity limiting factor [5]. However, the sensitivity limitation caused by non-specific binding still remains. Although the large size and the uneven shape of the particulate labels cause additional issues to the performance of immunoassay [3,6], like steric hindrance and increased non-specific binding, which reduce reproducibility, they enable increased signal from a single reporter. The ability to get more signal from a single fluorescent label without the use of higher excitation power leads to an improved detectability of the label and potentially more sensitive assay. In fluorescence based assays the use of higher excitation power would increase equivalently both fluorescence signal and background caused by autofluorescence and non-specific binding. In addition, in almost every case higher excitation power leads to more efficient photobleaching in imaging and in vivo experiments also to sample damage. Further, the particulate labels with high specific activity facilitate the use of simple instrumentation [7].

The most successful particulate reporters include for example quantum dots, lanthanide nanoparticles and up-converting

* Corresponding author. Tel.: +358 2 2678145.
E-mail address: telle.ukonaho@utu.fi (T. Ukonaho).

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phosphor particles (UCP-particles). Quantum dots, also called nanocrystals, have many unique properties such as small size (2–10 nm), broad excitation spectrum, adjustable emission wavelength with narrow emission band and good photostability. These particulate reporters have considerable number of applications mainly in the field of biological imaging [8–10]. Quantum dots have also some drawbacks. The use of this particulate label is limited by their low solubility and also by autofluorescence originating from excitation at ultraviolet light [9]. Lanthanide nanoparticles include both inorganic down-converting phosphor particles [11] and organic lanthanide chelate dyed polystyrene particles that may consist tens of thousands of lanthanide chelates in a single polystyrene particle [3]. These particulate labels have been successfully utilized both in heterogeneous and homogeneous immunoassays and in biological imaging [4,12–14]. Lanthanide chelates have their own characteristic properties that are also utilized in lanthanide nanoparticles. Long fluorescence lifetime enables time-resolved detection and thus eliminates background originating from autofluorescence. In addition, large Stokes' shift of lanthanide chelates enables simple separation of emission from excitation wavelengths. Lanthanide chelates are also relatively photostable and are not susceptible for self-quenching [15].

Among particulate labels, UCP-particles are a relatively new type of reporter that has shown enormous potential in recent studies [16,17]. UCP-particles are submicron sized inorganic crystals that have rare earth atoms (lanthanides; erbium and ytterbium) embedded in ceramic crystal structure [18]. UCP-particles are excited with infrared light (IR-light; low energy) and emit photons in the visible range (high energy). Compounds in biological samples cannot up-convert and thus, excitation in the IR-range does not produce autofluorescence from the sample in the visible range. Moreover, UCP-particles are commonly considered to be potentially photostable [18–20]. The ability to use higher IR-illumination intensities without autofluorescence, photobleaching of UCP-particles or sample damage translates into larger specific signals, and hence more sensitive assays. UCP-particles have also large Stokes' shift (in this case called anti-Stokes' shift; over 150 nm), narrow emission band and provide very long fluorescence lifetime [20,21]. Moreover, relatively cheap laser diodes in the excitation wavelengths of UCP-particles are available.

The irreversible destruction of an excited fluorophore, known as photobleaching has been studied with nanocrystals [22], lanthanide chelate dyed nanoparticles [14], inorganic down-converting lanthanide phosphors [11] and UCP-particles [18]. Most organic molecules including lanthanide chelates are susceptible to photobleaching. On the contrary, inorganic lanthanide phosphors are not susceptible to photobleaching because down- and up-conversion processes occurs within the inorganic host crystal [11,23]. Without decrease in their light emitting efficiency, these particles allow continuous excitation and repetitive analysis. However, the down-converting lanthanide phosphors are excited in the ultraviolet range and thus require time-resolved detection to avoid significant autofluorescence and instrumental background. Ultraviolet radiation is also harmful to biological material and may cause damage to the sample and this can change results obtained in biological imaging applications.

The excitation of inorganic UCP-particles in the IR-range causes no autofluorescence in the lower visible range wavelengths, where the emission of these particles is detected. The unique properties of UCP-particles have previously been utilized in many different applications, such as immunohistochemistry [24], immunochromatography [20], immunoassays [19,25], DNA hybridisation assays [23], microarray [26], DNA amplification assays [27] and in homogeneous assays [28]. Despite the fact that there has recently been a great interest towards UCP-particles, it is difficult to find evaluations where UCP-particles are compared with other particulate reporters with very high specific activity. In a previous study we have shown that both UCP-particles and lanthanide chelates provide excellent detectability in a simple model assay [29].

In the present work we have constructed two identical non-competitive two-site immunoassays using either UCP-particles or europium nanoparticles as labels. Prostate-specific antigen (PSA) was utilized as a model analyte in the assay. The aim of this work was to compare UCP-particles with Eu(III)-nanoparticles which are known to enable very sensitive immunoassays where the major limiting factor in sensitivity has stated to be non-specific binding. Additional objective in this work was to verify the common assumption of the photostability of UCP-particles supporting their potential applicability in imaging.

2. Experimental

2.1. Reagents

Clear MaxiSorp and white FluoroNunc MaxiSorp microtitration wells were purchased from NUNC (Roskilde, Denmark) and white 96-well plates from Greiner Bio-One International AG (Kremsmünster, Austria). Bovine serum albumin (BSA) fraction V was purchased from Bioreba (Nyon, Switzerland). Streptavidin was purchased from BioSpa, Società Prodotti Antibiotici (Milan, Italy). PSA free/total kit calibrators were obtained from Perkin Elmer Life and Analytical Sciences (Wallac Oy, Turku, Finland). Additional free-PSA calibrators were prepared by diluting the free-PSA calibrator to zero calibrator (50 mmol L^{-1} Tris–HCl, pH 7.75, containing 75 g L^{-1} BSA, 9 g L^{-1} NaCl and 0.5 g L^{-1} NaN_3). Assay buffer (50 mmol L^{-1} Tris–HCl, pH 7.8, containing 9 g L^{-1} NaCl, 0.5 g L^{-1} NaN_3, 5 g L^{-1} BSA, 0.1 g L^{-1} Tween 40, 0.5 g L^{-1} bovine γ-globulin and 20 μmol L^{-1} DTPA) and wash solution (5 mmol L^{-1} Tris–HCl, pH 7.8, containing 9 g L^{-1} NaCl, 0.05 g L^{-1} Tween 20, and 1 g L^{-1} Germall II) was purchased from Innotrac Diagnostics Oy (Turku, Finland). Poly(acrylic acid) Additol XW330 was purchased from Surface Specialties Austria GmbH (Wendorf, Austria). Free-PSA-specific monoclonal antibody 5A10 (Mab5A10) and total-PSA-recognizing monoclonal antibody H117 (MabH117) [30,31], recognizing non-overlapping epitopes on free-PSA, were produced in our laboratory (University