Analytical Biochemistry 446 (2014) 82–86

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Extension of dynamic range of sensitive nanoparticle-based immunoassays

Heidi Hyytiä [a,*], Noora Ristiniemi [a], Päivi Laitinen [b], Timo Lövgren [a], Kim Pettersson [a]

[a] Department of Biotechnology, University of Turku, 20520 Turku, Finland
[b] HUSLAB, Department of Clinical Chemistry, Helsinki University Hospital, 00029 HUS Helsinki, Finland

ARTICLE INFO

Article history:
Received 16 July 2013
Received in revised form 22 October 2013
Accepted 29 October 2013
Available online 6 November 2013

Keywords:
Immunoassay
Nanoparticle
Dynamic range
Desensitization

ABSTRACT

Nanoparticles have successfully been employed in immunometric assays that require high sensitivity. Certain analytes, however, require dynamic ranges (DRs) around a predetermined cut-off value. Here, we have studied the effects that antibody orientation and addition of free solid-phase and detection antibodies have on assay sensitivity and DR in traditional sandwich-type immunoassays. D-dimer and cardiac troponin I (cTnI), both routinely used in critical care testing, were applied as model analytes. The assays were performed in microtitration wells with preimmobilized solid-phase antibody. Inherently fluorescent nanoparticles coated with second antibody were used to detect the analyte. The selection of antibody orientation and addition of free solid-phase or detection antibody, with nanoparticles and calibrator, desensitized the assays and extended the DR. With D-dimer the upper limit of the DR was improved from 50 to 10,000 ng/ml, and with cTnI from 25 to 1000 ng/ml. Regression analysis with the Stago STA Liatest D-dimer assay yielded a slope (95% confidence interval) of 0.09 (0.07–0.11) and a *y*-intercept of −7.79 (−17.87–2.29) ng/L ($n = 65$, $r = 0.906$). Thus it is concluded that Europium(III)-chelate-doped nanoparticles can also be employed in immunoassays that require wide DRs around a certain cut-off limit.

Time-resolved fluorimetry of lanthanide chelate labels has established a distinguished role in bioaffinity assays during the past years [1]. Commercially available polystyrene beads containing >30,000 europium chelates, in combination with a derivatized surface, enabling immobilization of large numbers of binder reagents, have created exceptionally powerful detector modalities to achieve highly sensitive assays using simple test designs [2,3]. An ultimate illustration of this technology is that a single nanoparticle can be detected from a solution [2]. The wide applicability of Eu nanoparticles has been demonstrated in numerous bioaffinity assays for protein- [4], nucleic acid- [5], and virus-based diagnostics [6].

While the Eu-nanoparticle approach has provided ample evidence for exquisite analytical immunoassay sensitivities, the aim of this study was to investigate different approaches for the desensitization and extension of the dynamic range (DR)[1] of Eu-nanoparticle-based assays, while still using a conventional sandwich-type test approach. D-dimer and cardiac troponin I (cTnI), both commonly used critical care analytes, were selected as model analytes. Unlike cTnI, D-dimer requires a wide DR around a clinically predetermined comparatively high cut-off value. Hence, the analyte calls for test approaches capable of combining simplicity, speed, specificity, and a wide DR. The functionality of the D-dimer assay was tested by measuring a panel of patient citrated plasma samples with the desensitized novel assay and the results were compared to a commercially available immunoturbidimetric assay.

Materials and methods

D-dimer and cTnI calibrators and clinical samples

The D-dimer calibration material was prepared from partially purified D-dimer from human fibrin digested with human plasmin (Biokit, Barcelona, Spain), and human cTnI (native, tissue-derived cTnI–cardiac troponin T–troponin C complex) was purchased from HyTest (Turku, Finland). The calibrators were diluted in sample buffer containing 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4, and 10 g/L bovine serum albumin (BSA) (pH 7.2). Citrated leftover plasma samples ($n = 65$) were randomly collected at Oulu University Hospital (Oulu, Finland). The frozen samples were thereafter shipped to the University of Turku on dry ice and stored frozen at −70 °C. Prior to the analysis, the

* Corresponding author. Fax: +358 2 333 8050.
E-mail address: heidi.hyytia@utu.fi (H. Hyytiä).

[1] *Abbreviations used:* BSA, bovine serum albumin; cTnI, cardiac troponin I; CLSI, Clinical Laboratory Standards Institute; DR, dynamic range; FEU, fibrinogen equivalent units; F(ab′)$_2$, fragment antigen binding; LoB, limit of blank; SAv, streptavidin.

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.10.034

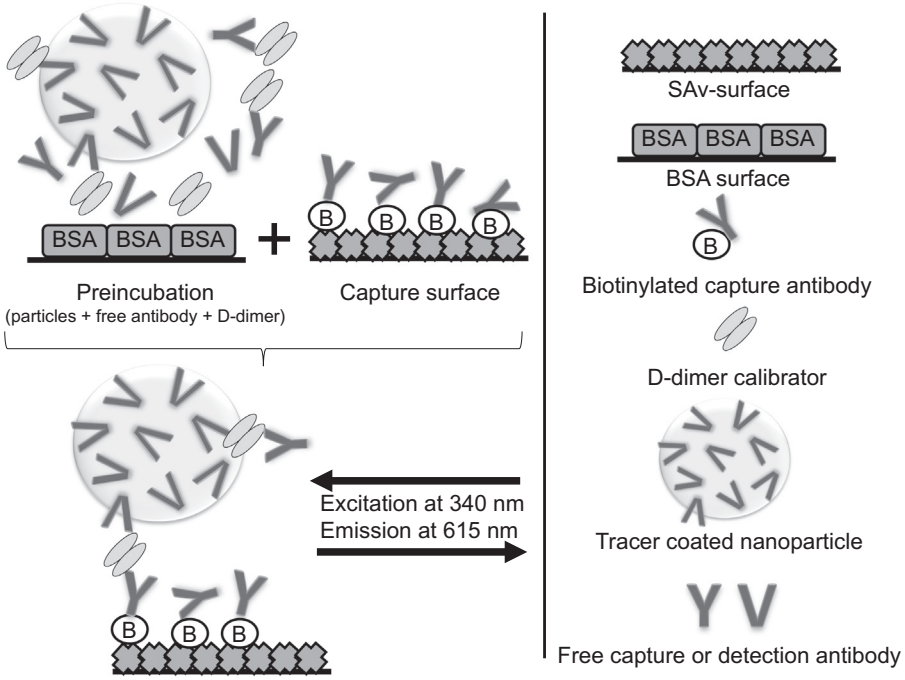

Fig.1. The principle of the developed sandwich-type modified one-step nanoparticle-based immunoassay for D-dimer using monoclonal FDP14 as the solid phase and 8D3F(ab′)$_2$ as the detection antibody attached to nanoparticles. The biotinylated monoclonal solid-phase antibody was immobilized into SAv-coated microtitration wells. Sample (12 μl) was applied simultaneous with europium(III)-chelate doped nanoparticles and free solid-phase or detection antibody (48 μl) to BSA-coated microtitration wells, and the solution was mixed for a 30-s preincubation. After the preincubation, 50 μl of the solution was moved to the solid-phase surface and the assay was incubated for 5 min at 23 °C. After the incubation, the wells were washed and europium fluorescence was measured in a time-resolved mode (not to scale).

frozen samples were thawed at +23 °C, mixed, and centrifuged (1 min, 2000*g*) to remove any particulate material.

Biotinylation of antibodies and preparation of solid-phase surfaces

A monoclonal antibody that specifically recognizes degraded forms of human fibrin and fibrinogen but not intact fibrin and fibrinogen (FDP14) and a monoclonal fragment antigen binding (F(ab′)$_2$) of antibody that specifically recognizes the D-dimer domain contained in the cross-linked degradation products of human fibrin (8D3) were from Biokit. Monoclonal cTnI-specific antibody 8I7 was purchased from International Point of Care (Toronto, ON, Canada) and monoclonal antibody 9707 was purchased from Medix Biochemica (Kauniainen, Finland). The solid-phase antibody FDP14 was biotinylated for 1 h at +23 °C, protected from light, in 50 mmol/L Na_2CO_3/$NaHCO_3$ buffer (pH 9.6) containing 150 mmol/L NaCl with 10-fold molar excess of EZ-Link NHS-Chromogenic-Biotin (Thermo Scientific, Rockford, IL, USA). The solid-phase antibody 8D3F(ab′)$_2$ was biotinylated for 1 h at +23 °C, protected from light, in 100 mmol/L phosphate buffer (pH 7.2) containing 150 mmol/L NaCl with 5-fold molar excess of EZ-Link NHS-Chromogenic-Biotin. Both biotinylated antibodies were purified with NAP-10 and PD-10 gel-filtration columns (GE Healthcare, Schenectady, NY, USA) by using 50 mmol/L Tris–HCl (pH 7.75), containing 150 mmol/L NaCl and 0.5 g/L NaN_3. The labeling degree for the FDP14 was 3 and for 8D3F(ab′)$_2$ 2 biotins per antibody. The labeled antibodies were stabilized with 1 g/L BSA (Bioreba, Nyon, Switzerland) and stored at +4 °C. The cTnI-specific antibodies were labeled with 10-fold (9707) and 20-fold (8I7) molar excess of biotin isothiocyanate using a procedure described earlier [7].

The assays were performed in streptavidin (SAv)-coated microtiter wells. Biotinylated solid-phase antibody (120 ng) was immobilized to SAv-coated wells in 60 μl of assay buffer (Kaivogen buffer solution red; Kaivogen Oy, Turku, Finland). After 1 h incubation at +23 °C and shaking at 900 rpm, the wells were washed four times with wash solution (Kaivogen wash concentrate; Kaivogen Oy) and used immediately in the assay.

Conjugation of antibodies to detector nanoparticles

Nanoparticles were purchased from Seradyn (Indianapolis, IN, USA) and were internally dyed, monodisperse, carboxyl-modified Fluoro-Max polystyrene, 107-nm particles (carboxyl content 0.157 mEq/g, parking area 56.6 Å^2), which produce a long-lifetime fluorescence equivalent to 30,000 chelated ions per particle [2]. Primary amino groups of FDP14 and 8D3F(ab′)$_2$ were covalently coupled to activated carboxyl groups of the nanoparticles using a procedure described previously with some minor modifications [6]. The nanoparticles (1.5×10^{12} particles) were suspended in 10 mmol/L phosphate buffer (pH 7.0), and their surfaces were activated with 0.75 mmol/L *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (Sigma–Aldrich, St. Louis, MO, USA) and 10 mmol/L *N*-hydroxysulfosuccinimide sodium salt (Sigma–Aldrich). The concentrations of FDP14 and 8D3F(ab′)$_2$ in the coupling reactions were 1.0 and 0.8 mg/ml, respectively, and the reactions contained 100 mmol/L NaCl. The activated particles were mixed either directly (8D3F(ab′)$_2$) or dropwise (FDP14) with the antibodies. The coupling reactions were incubated for either 50 min (FDP14) or 2 h (8D3F(ab′)$_2$) at +23 °C under vigorous shaking. Final washes and blocking of the remaining active groups were performed in Tris-based buffer (10 mmol/L Tris, 0.5 g/L NaN_3, pH 8.5), and the nanoparticle–antibody conjugates were stored in the same buffer supplemented with 2 g/L BSA at 4 °C. Before the first instance of use, the particles were mixed thoroughly, sonicated, and centrifuged lightly (350*g*, 5 min) to separate noncolloidal aggregates from the monodisperse suspension. The cTnI-specific conjugates were prepared as described previously [8].