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Extension of dynamic range of sensitive nanoparticle-based immunoassays

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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

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[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.

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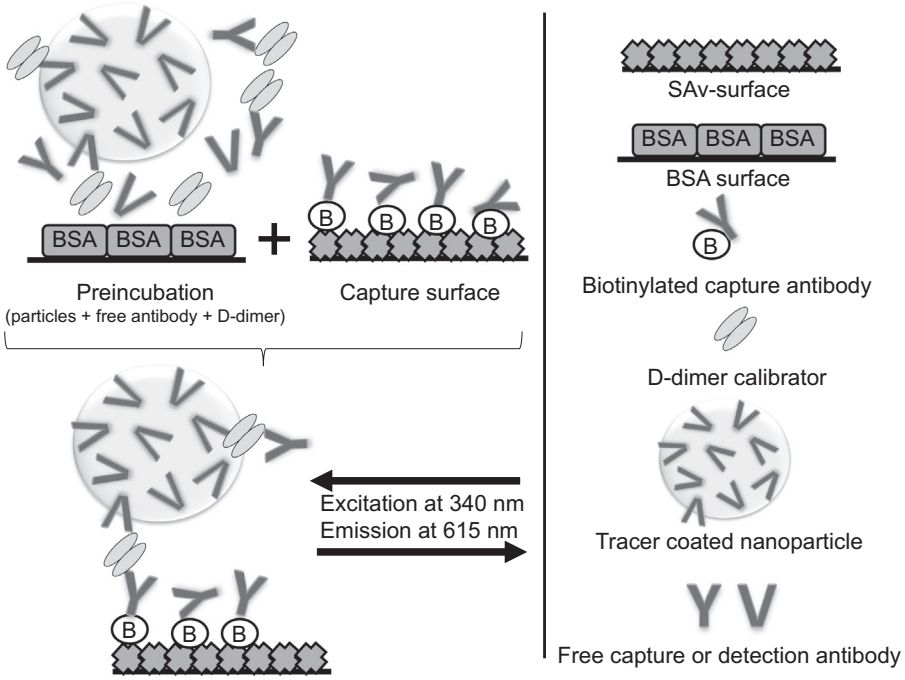

Fig.1. The principle of the developed sandwich-type modified one-step nanoparticle-based immunoassay for D-dimer using monoclonal FDP14 as the solid phase and 8D3F(ab′)$_2$ as the detection antibody attached to nanoparticles. The biotinylated monoclonal solid-phase antibody was immobilized into SAv-coated 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].

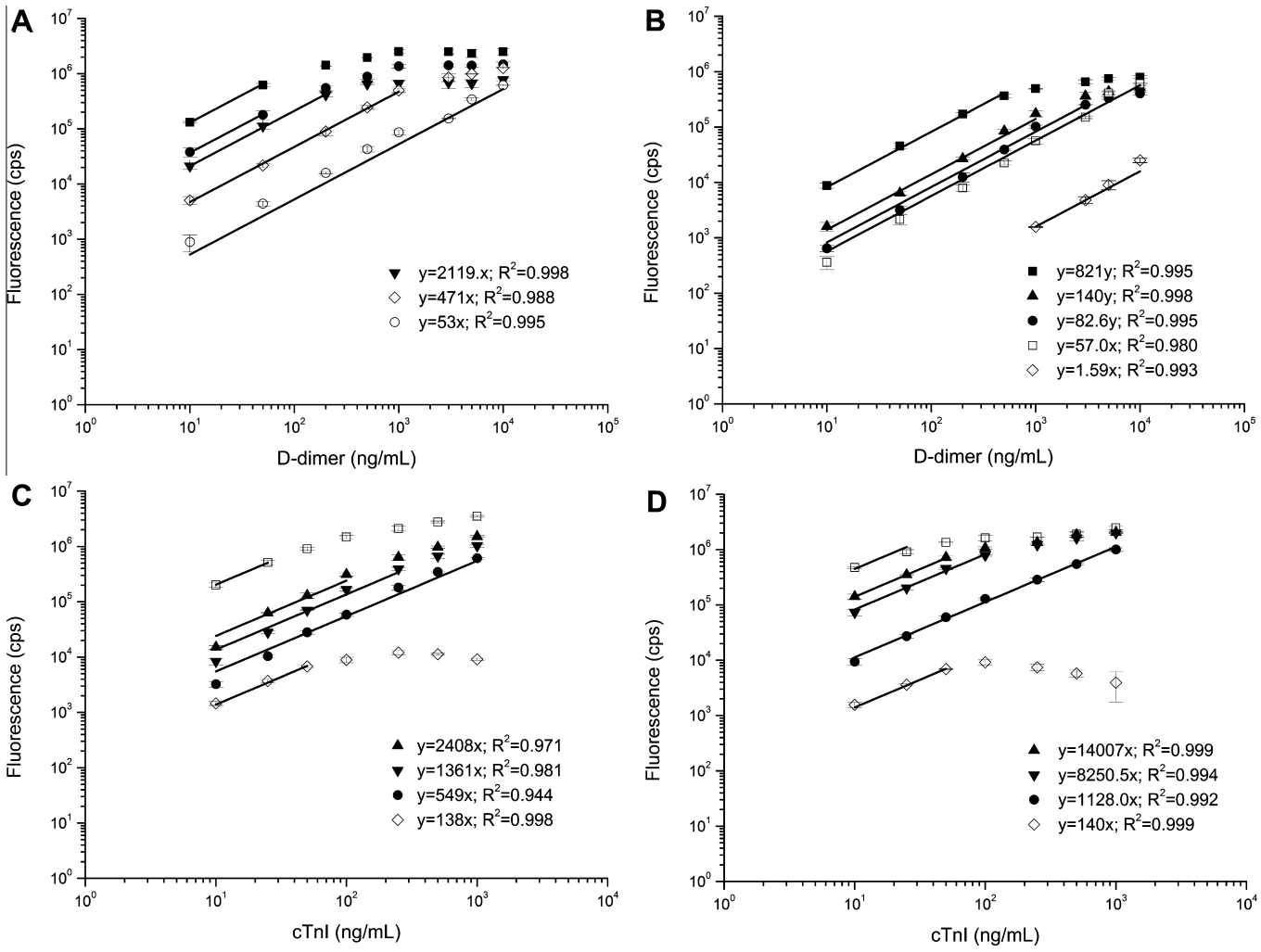

Fig.2. Dose–response curves for the D-dimer and cTnI immunoassays. The error bars represent the standard deviation measured from three replicate microtitration wells. (A) The D-dimer assay exploiting 8D3F(ab′)$_2$ as the solid-phase and FDP14 as the detection antibody. (■) 0 μg/ml free FDP14/8D3F(ab′)$_2$, (●) 5 μg/ml free 8D3F(ab′)$_2$, (▼) 15 μg/ml free 8D3F(ab′)$_2$, (◇) 5 μg/ml free FDP14, and (○) 15 μg/ml free FDP14. (B) The D-dimer assay exploiting FDP14 as the solid-phase and 8D3F(ab′)$_2$ as the detection antibody. (■) 0 μg/ml free DP14/8D3F(ab′)$_2$, (▲) 5 μg/ml free 8D3F(ab′)$_2$, (●) 15 μg/ml free 8D3F(ab′)$_2$, (□) 1 μg/ml free FDP14, and (◇) 5 μg/ml free FDP14. (C) The cTnI assay exploiting 8I7 as the solid-phase and 9707 as the detection antibody. (□) 0 μg/ml free 8I7/9707, (▲) 5 μg/ml free 9707, (▼) 10 μg/ml free 9707, (●) 25 μg/ml free 9707, and (◇) 10 μg/ml free 8I7. (D) The cTnI assay exploiting 9707 as the solid-phase and 8I7 as the detection antibody. (□) 0 μg/ml free 8I7/9707, (▲) 5 μg/ml free 8I7, (▼) 10 μg/ml free 8I7, (●) 25 μg/ml free 8I7, and (◇) 10 μg/ml free 9707.

Nanoparticle-based immunoassay design

The D-dimer-specific nanoparticle-based immunoassays were developed by employing FDP14 as the solid phase and 8D3F(ab′)$_2$ as the detection antibody (Fig. 1) or vice versa. The cTnI assays were developed identical to the D-dimer assays, but employing 8I7 and 9707 antibodies. The immunoassays were performed as modified one-step sandwich-type formats with preincubation of the calibrator, free solid-phase or detection antibody, and antibody-coated detection nanoparticles prior to presentation to the specific solid-phase surface. The preincubation step was added to simulate the usage of simple microfluidic chip processing, in which sample and detector are first introduced and thereafter transferred to the solid-phase surface.

First 1.44×10^9 (1.8×10^8 for cTnI assays) detection antibody-coated nanoparticles along with 0–25 μg/ml free solid-phase or detection antibodies were added in 48 μl of particle buffer (assay buffer supplemented with 0.1 g/L native mouse IgG, 0.05 g/L denatured mouse IgG; Meridian Life Science, Saco, ME, USA) to wells that were coated with 5 g/L BSA. The samples or calibrators were added in 12 μl, and the solution was preincubated for 30 s at +23 °C with shaking at 900 rpm. After the preincubation in BSA-coated wells, 50 μl of the calibrator-particle solution was transferred to the solid-phase surface. The assay wells were then incubated for 5 min at +23 °C with shaking at 900 rpm. Finally, the wells were washed thoroughly with wash solution and long lifetime fluorescence of the bound nanoparticle bioconjugates was measured directly from the surface of the well in a time-resolved mode with a Victor 1420 multilabel counter (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland).

Assay desensitization and evaluation

The extension of D-dimer assay DR was studied by supplementing 0–15 μg/ml free solid-phase or detection antibody into the particle buffer when 8D3F(ab′)$_2$ was used as the detection antibody and FDP14 as the solid-phase antibody. When the two antibodies were in reversed order (FDP14 as the solid-phase and 8D3F(ab′)$_2$ as the detection antibody), 0–15 μg/ml free 8D3F(ab′)$_2$ and 0–5 μg/ml free FDP14 were tested. When cTnI was used as the analyte, 0 and 10 μg/ml free solid-phase antibody and 0–25 μg/ml free detection antibody were applied. The DRs of the

assays were determined by observing the range of output signal that was a linear function of the input calibrator amount. Limit of blank (LoB) was determined according to Clinical Laboratory Standards Institute (CLSI) Guideline EP17-A (n = 12), as determined with the nonparametric option [9].

Method comparison

The performance parameters of the developed D-dimer immunoassay (FDP14 as the solid-phase and 8D3F(ab′)$_2$ as the detection antibody, with 1 μg/ml free FDP14) were tested (with 15-min incubation) against a commercially available quantitative immunoturbidimetric latex agglutination STA Liatest D-dimer assay (Diagnostica Stago, Parsippany, NJ, USA) with a Stago STA-R Evolution device (Diagnostica Stago). The comparison assays were performed at the University Hospital of Oulu according to the manufacturer's instructions. The results of the comparison assay were given as ng/ml fibrinogen equivalent units (FEU), and a D-dimer result of ⩾500 ng/ml (FEU) was considered positive. Assay descriptives and Pearson correlation was calculated with IBM SPSS Statistics 21 (SPSS, Inc., Chicago, IL, USA). Method comparison was performed by calculating Deming regression parameters with Analyze-It software (Analyze-It Software Ltd., Leeds, UK).

Results and discussion

The principle of the developed D-dimer immunoassay can be seen in Fig. 1. The antibodies were tested with FDP14 (8I7, cTnI) as the solid-phase and 8D3F(ab′)$_2$ (9707, cTnI) as the detection antibody attached to nanoparticles or vice versa. The assays were performed as a modified one-step format in which the analyte, antibody-coated detection particles, and free antibody were preincubated for 30 s in BSA-coated wells and thereafter transferred to a solid-phase antibody-coated capture surface. The preincubation step was employed, since the ultimate goal is to use the assay in a simple microfluidic chip, where the calibrator and detection nanoparticles are first introduced and later transferred to a solid-phase surface. Extension of DR was expected, as the free antibody competes for the binding to the analyte with the solid-phase and the detection antibodies, respectively.

Typical calibration curves for the developed assays are shown in Fig. 2A and B for D-dimer and Fig. 2C and D for cTnI. When the 8D3F(ab′)$_2$/FDP14 solid-phase/detection configuration was employed in the D-dimer assay, addition of 15 μg/ml free solid-phase antibody (8D3F(ab′)$_2$) widened the upper limit of DR of the assay 4-fold, from 50 to 200 ng/ml (Table 1). Addition of free detection antibody (FDP14) had a clearly more significant effect on DR: addition of 15 μg/ml free FDP14 extended the upper limit of DR 200-fold to 10,000 ng/ml. When antibodies were employed in the reverse configuration (FDP14/8D3F(ab′)$_2$), the DR was ⩽500 ng/ml without the addition of free antibodies. Addition of free detection antibody (8D3F(ab′)$_2$) had a moderate effect on the DR; addition of 15 μg/ml free detection antibody increased the upper limit of DR 6-fold from 500 to 3000 ng/ml. Addition of 1 or 5 μg/ml free solid-phase antibody (FDP14) increased the DR 20-fold to 10,000 ng/ml. Thus, regardless of the antibody orientation in the assay, addition of free FDP14 had clearly better effects on shifting the assay DR than the addition of free 8D3F(ab′)$_2$. With the FDP14/8D3F(ab′)$_2$ configuration the amounts of free FDP14 required to shift the DR were significantly smaller than with the reverse antibody orientation. No high-dose hook was observed in the assays, and the signals were still increasing at 10,000 ng/ml, which was the highest D-dimer concentration measured. The lowest D-dimer concentration tested was 10 ng/ml, although lower concentrations would have been in the linear range for many of the tested assay versions. The widely used clinical cut-off level of D-dimer is 500 ng/ml (FEU) [10]. For cTnI, both assay configurations reacted very similar to the antibody additions: the addition of 10 μg/ml free solid-phase antibody increased the upper limit of DR 2-fold from 25 to 50 ng/ml (Table 1). The addition of 10 μg/ml free solid-phase antibody also resulted in a high-dose hook: most clearly seen with the 9707/8I7 configuration. The addition of 25 μg/ml free detection antibody increased the upper limit of DR 40-fold to 1000 ng/ml with both configurations.

Table 1
The calculated limit of blank (LoB) and upper limit of dynamic range (DR) of the various assay configurations tested

	LoB (ng/ml)	DR (ng/ml)
D-dimer		
8D3F(ab′)$_2$–FDP14		
0 μg/ml free FDP14/8D3F(ab′)$_2$	0.249	⩽50
5 μg/ml free 8D3F(ab′)$_2$ (solid phase)	0.463	⩽50
15 μg/ml free 8D3F(ab′)$_2$ (solid phase)	0.158	⩽200
5 μg/ml free FDP14 (detection)	2.73	⩽1000
15 μg/ml free FDP14 (detection)	13.6	⩽10,000
FDP14–8D3F(ab′)$_2$		
0 μg/ml free FDP14/8D3F(ab′)$_2$	1.17	⩽500
1 μg/ml free FDP14 (solid phase)	2.75	⩽10,000
5 μg/ml free FDP14 (solid phase)	478	⩽10,000
5 μg/ml free 8D3F(ab′)$_2$ (detection)	0.331	⩽1000
15 μg/ml free 8D3F(ab′)$_2$ (detection)	0.603	⩽3000
Cardiac troponin I		
8I7–9707		
0 μg/ml free 8I7/9707	0.0100	⩽25
10 μg/ml free 8I7 (solid phase)	1.03	⩽50
5 μg/ml free 9707 (detection)	0.0356	⩽100
10 μg/ml free 9707 (detection)	0.0808	⩽250
25 μg/ml free 9707 (detection)	0.0367	⩽1000
9707–8I7		
0 μg/ml free 8I7/9707	0.00300	⩽25
10 μg/ml free 9707 (solid phase)	1.06	⩽50
5 μg/ml free 8I7 (detection)	0.00850	⩽50
10 μg/ml free 8I7 (detection)	0.0154	⩽100
25 μg/ml free 8I7 (detection)	0.290	⩽1000

LoBs, determined according to CLSI Guideline EP17-A, can be seen on Table 1. With the 8D3F(ab′)$_2$/FDP14 configuration the LoB was <1 ng/ml. Employing the second antibody orientation (FDP14/8D3F(ab′)$_2$) resulted in an approximately fivefold (1.17 ng/ml) less sensitive assay, but produced a wider linear range and shifted the range toward higher concentrations (0.249–50 ng/ml vs 1.17–500 ng/ml). For cTnI and the 8I7/9707 configuration, the LoB was 0.0100 ng/ml. Employing the reverse antibody orientation (9707/8I7) resulted in a threefold less sensitive assay (0.300 ng/ml). With the 8I7/9707 configuration, the addition of free detection antibody (9707) did not affect the LoB as remarkably as with the 9707/8I7 configuration and the addition of free detection antibody (8I7). The addition of 25 μg/ml free detection antibody with the 9707/8I7 configuration resulted in an eightfold more sensitive assay than with the opposite configuration (0.0367 vs 0.290). As the amount of the free antibody increased, so did the LoB in the majority of assay versions. As the amount of free antibody was increased, fewer antigens were able to bind both solid-phase and detection antibodies, thus resulting in assay desensitization. Consequently the antibody orientation itself in two-site immunoassays may have significant effects on the assay sensitivity and positioning of the DR.

Extending the assay DR in immunoassays is traditionally approached by trying to achieve an assay that is as sensitive as possible and allows small sample volumes to be used [11]. The use of the highest possible excesses of functionally active binders serves the purpose of providing an opportunity for high DRs in combination with light-producing reporter techniques [12]. The use of excess solid-phase and detector reagents also serves to shorten

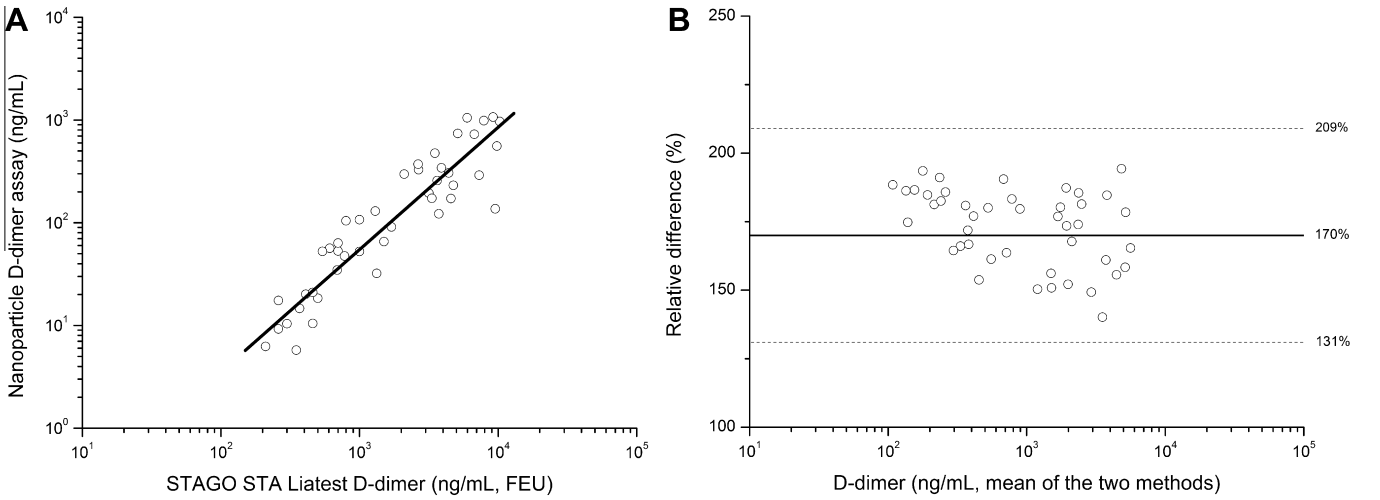

Fig.3. Method comparison. (A) Correlation between the developed D-dimer assay and STA Liatest D-dimer assay. ($n = 65$, $r = 0.906$) (B) Bland–Altman analysis of agreement between the two methods. Solid horizontal line represents the mean difference and dotted lines the 95% limits of agreement.

the incubation time and theoretically also provides higher assay sensitivities assuming that the nonspecific binding can be controlled [13]. Using multiple antibodies with differing affinities simultaneously has also been used to obtain extended DRs: an approach that requires multiple additional steps [14]. Novel ways to enhance the DR in protein detection assays include the use of specifically labeled fluorescence immunosensors [15] and the use of plasmonic structures and molecular spacers that enhance the DR up to 8 orders of magnitude in comparison to traditional assays [16].

The applicability of the developed D-dimer assay to measure plasma D-dimer was investigated by measuring 65 citrated plasma samples left over from hospital routine analysis. Using an assay time (5 min) in which the signal is rapidly developing increases the possibility of between-run imprecision. Thus a 15-min incubation time was selected when analyzing the plasma samples. The median concentration of D-dimer (lower and upper quartiles) was 1700 ng/ml (575 and 4485 ng/ml, FEU) when measured with the immunoturbidimetric comparison assay, whereas the median D-dimer concentration measured with the developed nanoparticle-based assay was 111 ng/ml (36.5 and 354 ng/ml). The regression analysis yielded a slope (95% confidence intervals) of 0.09 (0.07–0.11) and a y-intercept of −7.79 (−17.87–2.29) ng/L. The Spearman correlation coefficient was 0.906 ($P < 0.0001$). The mean relative difference between the two methods was 170% with the 95% limits of agreement ranging from 209 to 131% (Fig. 3).

Conclusions

Desensitization and extension of DRs for nanoparticle-based assays for D-dimer and cTnI were performed by selecting the correct antibody configuration and addition of free solid-phase or detection antibodies. For cTnI, the addition of free solid-phase antibody had little effect on assay LoB and DR. With the addition of free detection antibody, an extension of the upper limit of DR by 40-fold was achieved. For D-dimer, the selection of correct antibody configuration (FDP14/8D3F(ab′)$_2$) and addition of 1 μg/ml free solid-phase antibody was enough to show an extension of the upper limit of DR by 200-fold. Hence, europium(III)-chelate doped nanoparticles can be exploited both in immunoassays requiring high sensitivities and in immunoassays that require wide DR around a specific, nondemanding analyte concentration.

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