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Supersensitive photon upconversion based immunoassay for detection of cardiac troponin I in human plasma

Kirsti Raiko *, Annika Lyytikäinen, Miikka Ekman, Aleksi Nokelainen, Satu Lahtinen, Tero Soukka

Department of Biotechnology, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland

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ABSTRACT

Background and aims: Upconverting nanoparticles (UCNPs) are attractive reporters for immunoassays due to their excellent detectability. Assays sensitive enough to measure baseline level of cardiac troponin I cTnI in healthy population could be used to identify patients at risk for cardiovascular disease. Aiming for a cTnI assay of such sensitivity, the surface chemistry of the nanoparticles as well as the assay reagents and the protocol were optimized for monodispersity of the UCNP antibody conjugates (Mab UCNPs) and to minimize their non-specific interactions with the solid support.

Materials and methods: UCNPs were coated with poly(acrylic acid) via two-step ligand exchange and conjugated with monoclonal antibodies. The conjugates were applied in a microplate-based sandwich immunoassay using a combination of two capture antibodies to detect cTnI. Assay was evaluated according to guidelines of Clinical & Laboratory Standards Institute.

Results: The limit of detection and limit of blank of the assay were 0.13 ng/L and 0.01 ng/L cTnI, respectively. The recoveries were >90% in spiked plasma in the linear range. The within- and between-run imprecisions were <10%.

Conclusion: The results demonstrate that UCNPs enable quantification of cTnI concentrations expected in plasma of healthy individuals and could be used to identify patients at risk for cardiovascular disease.

1. Introduction

Upconverting nanoparticles (UCNPs) have various characteristics, which make them attractive luminescent reporters in biomolecule detection.[1] Unlike other photoluminescent materials, UCNPs can convert low energy near-infrared light to high energy light through sequential and cumulative absorption of photons, resulting in anti-Stokes shifted upconversion luminescence (UCL) emission.[2] This feature enables the spectral elimination of autofluorescence, making UCNPs highly detectable. The hardware required for the detection of UCNPs is also simple and affordable compared to, for example, use of time gated detection technologies for elimination of autofluorescence.[3] UCNPs also do not suffer from photobleaching,[4] and multiple antibodies can be conjugated to individual UCNPs resulting in kinetically enhanced binding affinity,[5] thus further improving sensitivity of detection.

The diagnosis of myocardial infarction relies heavily on the detection of cardiac troponin,[6] usually either I or T (cTnI or cTnT) subcomponents of the troponin complex, of which cTnI is the target of most current assays. The emergence of high sensitivity (hs) detection methods for cardiac troponin has enabled rapid diagnostics of myocardial infarction and shortened the emergency room waiting times, as smaller changes in troponin levels are detected and time interval between blood sampling is reduced.[6] Capability of measuring the baseline levels of cTnI also enables predicting the risk of future myocardial infarctions, but this level of sensitivity has only been sparsely reached with a few hs-cTnI assays.[7] In addition, there is novel evidence that quantitation of cardiac biomarkers can be used as a prognostic tool for evaluating the

Abbreviations: UCNPs, upconverting nanoparticles; UCL, upconversion luminescence; cTnI/-T, cardiac troponin I/T; hs, high sensitivity; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine; LoD, limit of detection; SD, standard deviation; PAA, poly(acrylic acid); ADMA, 2-amino-N,N-dimethylacetamide; DBU-1, 8-Diazabicyclo[5.4.0]-7-undecene; TEM, transmission electron microscopy; CLSI, Clinical & Laboratory Standards Institute; LoB, limit of blank.

* Corresponding author.
E-mail address: kisrai@utu.fi (K. Raiko).

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outcome in Covid-19 patients,[8] which further augments the need for hs-troponin assays.

Accurate comparison of troponin assays is made difficult by the lack of international standardization.[9] However, The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) has listed the analytical characteristics of current hs-cTnI assays as announced by the manufacturers, and the limits of detection (LoD) vary mostly between 1 and 5 ng/L cTnI.[9] The highest sensitivity among the assays mentioned in the list was achieved by the Singulex Clarity system (LoD 0.08 ng/L), which is based on single molecule counting, a niche technology involving a highly specialized assay platform. Application of UCL-technology for the detection of cTnI has resulted in LoDs as low as 1.68 ng/L[3] and 0.48 ng/L[10] cTnI (determined by 3 × standard deviation [SD] of zero calibrator) in heterogeneous sandwich assays on microtiter plates.

The main sensitivity limiting factors in UCL-assays have widely been considered to be non-specific binding of UCNP reporter conjugates and aggregation, especially in biological matrices, as discussed also in the above-mentioned publications. The greatest factor influencing the loss of monodispersity has been concluded to be the surface chemistry of UCNPs.[11,12] The issue of non-specific binding, however, requires fine tuning of all aspects of the assay and has been previously approached by adjusting buffer compositions[10] and pre-processing the biological sample to remove the components causing non-specific binding,[13] among others.

This article presents to our knowledge the most sensitive cTnI immunoassay in microtitration plate platform ever published. For the assay the UCNPs were coated with poly(acrylic acid) (PAA) via two-step ligand exchange process using $NOBF_4$ as the intermediate, followed by conjugation to monoclonal antibodies (Mab). The coating and conjugation procedures were optimized to improve monodispersity and reduce the non-specific binding to solid support. The heterogeneous sandwich assay was carried out in a standard 96-well microtiter plate format and did not involve complex sample pretreatment steps. The assay reagents and protocol were also optimized to improve the sensitivity of detection. The assay principle is presented in Fig. 1. Successful reduction of aggregation and non-specific binding of the reporter conjugate, as well as careful optimizations of the assay reagents and conditions, culminated in the achieved remarkable sensitivity. To validate the accuracy of the calibrators, a commercial hs-cTnI assay was used as a reference assay.

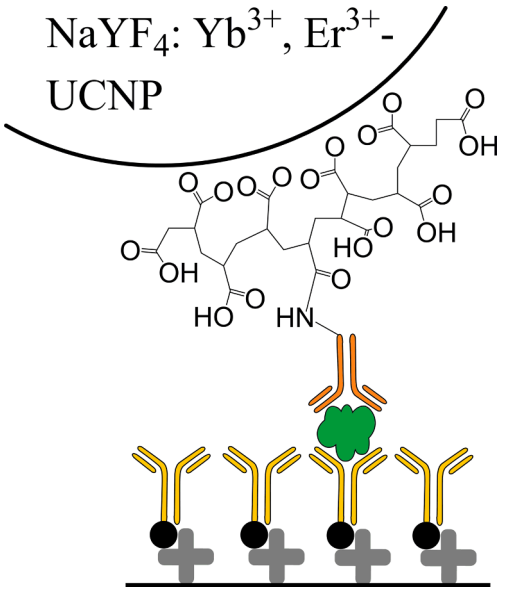

Fig. 1. Principle of the super-sensitive upconversion luminescence based immunoassay for the detection of cTnI. Streptavidin (grey) passively coated inside a microtiter well immobilizes biotinylated (black sphere) capture antibodies (yellow). The analyte cTnI (green) is detected by the antibody (orange) conjugated PAA-coated UCNPs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Experimental section

2.1. Materials and reagents

Upconverting nanoparticles were synthesized as described previously [14]. Monoclonal capture antibody clones 19C7 and 9707 (Mab 19C7 and Mab 9707) were from HyTest (Turku, Finland) and Medix Biochemica (Espoo, Finland), respectively, and were biotinylated as described previously [15]. Monoclonal antibody clone 625 (Mab 625) was purchased from HyTest, as well as the human cardiac troponin ITC-complex. Probumin BSA used for blocking the streptavidin plates and casein from bovine milk (Calbiochem) were purchased from Merck Millipore (Burlington, Massachusetts). BSA for other uses was from Bioreba AG (Reinach, Germany). Fat-free milk powder was purchased from Valio Oy (Helsinki, Finland). C8 Lockwell LUMI White Maxisorp microtiter plates were purchased from Thermo Scientific (Waltham, Massachusetts) and passively coated with 1 μg/well streptavidin (Biospa, Milan, Italy) as described previously [16]. The assay buffer and wash buffer were purchased from Kaivogen Oy (Turku, Finland). Mouse IgG was purchased from Meridian Life Science (Memphis, Tennessee). Denatured mouse IgG was prepared by incubation in 63 °C for 30 min. In addition, 2-amino-N,N-dimethylacetamide (ADMA) was purchased from Combi-Blocks (San Diego, California), D(+)-trehalose dehydrate from VWR (Radnor, Pennsylvania), and 1,8-Diazabicyclo[5.4.0]-7-undecene (DBU) from TCI (Tokyo, Japan), while all other reagents were purchased from Sigma Aldrich (Saint Louis, Pennsylvania). Blood for the plasma pool was collected from five anonymized healthy volunteers in lithium-heparin vacuum tubes (Vacuette® 9 ml, Greiner Bio-one, Kremsmünster, Austria) in compliance with the Helsinki agreement.

2.2. Surface modification of UCNPs

The size of oleic acid-capped $NaYF_4$: 17% Yb^{3+}, 3% Er^{3+} UCNPs in toluene was measured with transmission electron microscopy (TEM) imaging based technique described in the Supporting Information (SI). They were rendered water soluble by tetrafluoroborate (BF_4^-) ligand exchange, as described previously [17]. The BF_4-ligand was exchanged to PAA (Mw 2000) in two alternative ways, the first approach following the coating described previously [17]. In the second approach, a two-fold mass excess of PAA was added to the BF_4-coated UCNPs, followed by addition DBU to reach pH 9 (4 % [v/v] DBU in final reaction), and of DMF to adjust the concentration of PAA to 3.33 % (w/v) in the final reaction. The main difference between the methods was that in the first version, PAA was added to the reaction as a 10% (w/v) solution in NaOH-adjusted water of pH 9, instead of using DBU. Both reactions were incubated for 24 h in 60 °C at 1400 rpm rotation and washed as described previously [17]. The difference between use of DBU or NaOH in the pH adjustment of PAA-coating reaction of UCNPs was studied. The proportion of clustered (non-bioconjugated) PAA-UCNPs in the solution and their size distribution was evaluated on gel electrophoresis [18] following a protocol described previously [17]. After bioconjugation, the Mab-UCNPs were tested in a cTnI assay using 0.05–5000 ng/L cTnI calibrators, following a protocol also described previously [10]. The same mass of Mab-UCNPs was used, the only difference between the used Mab-UCNP solutions was the base used in the pH adjustment of the PAA-coating reaction. Sensitivities of the assays conducted with both coating methods were calculated from linear fit on Origin 8 (Origin Lab, Northampton, MA) of zero calibrator subtracted UCL signals based on 3 × SD of zero calibrator. UCL signal-to-background (zero calibrator) ratios were also calculated for all calibrator concentrations.

2.3. Bioconjugation of PAA-UCNPs

PAA-UCNPs were conjugated to Mab 625 as described previously [10] using EDC/sulfo-NHS chemistry in 20 mM 2-(N-morpholino)ethanesulfonic acid (MES)-buffer (pH 6.1), with a few modifications. First, in a conjugation reaction of 2 mg UCNPs in 250 μL, 30 mM of sulfo-NHS was used with either 2.5; 5; 10; 15; 20 or 30 mM of EDC. Secondly, the surface was blocked by adding ADMA (as 2 M solution in water, pH 11) to a final concentration of 50 mM in reaction and slowly rotating the tube at room temperature for 30 min. After washing, the antibody-conjugated UCNPs (Mab-UCNPs) were suspended in Mab-UCNP storage buffer (5 mM Tris, pH 8.5, 0.05 % Tween-85, 0.5 % BSA, 0,05% PAA (Mw 1200), 5% trehalose, 5% ethylene glycol and 0.05% NaN_3 [w/v]) and stored in +4 °C. Cluster abundance was studied in a filtration test, as described previously [17]. The Mab-UCNPs conjugated with different EDC to sulfo-NHS -ratios were incubated for 30 min in sample buffer with 20% (v/v) of either 7.5% (w/v) BSA-TSA or plasma, before filtration through 0.22 and 0.1 μm pore size syringe filters. The binding efficiency of the Mab-UCNPs, defined as analyte-bound fraction of the Mab-UCNPs added in the well, was evaluated in an activity assay [18]. cTnI calibrators of 50; 500 and 5000 ng/L in three replicates and 10 ng/well of Mab-UCNPs were used. The total UCL of Mab-UCNPs was measured from the wells at the start of the detection incubation and the UCL of analyte-bound Mab-UCNPs after the assay from dried well. The analyte bound fractions with 50 ng/L and 500 ng/L cTnI were multiplied with factors 100 and 10 (respectively) to scale them to correspond that of the 5000 ng/L calibrator, and the average binding efficiency was calculated.

2.4. Immunoassay protocol

The assay was modified from a previously published protocol [10]. The optimization of streptavidin brand, the pH of wash buffer after Mab-UCNP incubation in assay, type of 9707 capture antibody, proportion of sample (calibrator or spiked plasma pool) in sample buffer, along with the incubation time and concentration of Mab-UCNP in the assay, are described in detail in the SI. A Mab-UCNP dilution was prepared 30 min prior to starting the assay by diluting Mab-UCNPs to modified assay buffer (assay buffer supplemented with 1 mM KF, 0.05% PAA (Mw 1200), 0.2% fat-free milk powder, 0.08% native mouse IgG and 0.005% denatured mouse IgG [w/v]) to a concentration of 4.0 ng Mab-UCNPs/μL. The plate was prewashed and biotinylated anti-cTnI capture antibodies Mab 19C7 and Mab 9707 were immobilized to the wells (100 ng/well each in 50 μL assay buffer) by incubating 30 min in slow shaking at room temperature. The plate was washed once with wash buffer. The cTnI calibrators (zero calibrator and 0.05–50 000 ng/L cTnI) were prepared in 7.5% BSA-TSA (50 mM Tris, pH 7.75, 150 mM NaCl and 0.05% NaN_3, with 7.5% BSA, [w/v]), and diluted in sample buffer (37.5 mM Tris, pH 8, with 0.5 M NaCl, 0.06% bovine gamma-globulin, 2.5% BSA, 5% D-trehalose, 37.5 U/mL heparin, 0.08% native mouse IgG, 0.005% denatured mouse IgG, 0.2% casein, 0.0375% NaN_3, [w/v]), using 80% cTnI calibrator and 20% sample buffer (v/v). Plasma was centrifuged 3 000*g* for 10 min to remove precipitated plasma components and the supernatant was diluted to sample buffer similarly to the cTnI calibrators. The calibrators and plasma samples were pipetted 50 μL/well and incubated in slow shaking at room temperature for 30 min, followed by washing once. The Mab-UCNP dilution prepared earlier was sonicated with a vial tweeter sonicator (3 cycles, 0.5 s with 100% amplitude, Hielscher Ultrasonics GmBH, Germany) and pipetted 50 μL/well, incubated for 15 min at room temperature in slow shaking, after which the plate was washed four times and allowed to dry for approx. 45 min before measurement. Unlike all previous washes, the pH of the wash buffer was adjusted to 10.25 with NaOH for the last wash. Upconversion luminescence in the bottoms of the dry wells was measured with a 3x3 raster program (1.5 mm between points) at 540 nm using a modified Plate Chameleon microplate reader (Hidex Oy, Turku, Finland) equipped with a 980 nm laser [1].

2.5. Assay evaluation

Linear range of the assay was determined. Assay performance evaluation was conducted following the Classical Approach of Clinical & Laboratory Standards institute (CLSI) Guideline EP17-A2. Four blank samples (80% zero calibrator [7.5% BSA-TSA], 20% sample buffer) were assayed on four days and the limit of blank (LoB) was determined on a nonparametric analysis of the total 60 replicates of blank. For determination of the limit of detection, four low cTnI level plasma samples (80% non-spiked plasma, 20% sample buffer) were assayed as four replicates on four days, followed by parametric analysis of results of the total 64 replicates. The LoD was also calculated according to IUPAC-definition as 3 × SD of zero calibrator [19] and linear fit analysis with Origin 8 based on the same assays as the CLSI-based LoD calculations. Within- and between-run precisions were calculated for the standard curve for five assay repeats, where each concentration was measured in four replicates per assay. Recovery percentages were determined throughout the dynamic range by spiking pooled plasma and each concentration at the linear range was quantified as 4 replicates. The concentration of non-spiked plasma was reduced from the spiked plasma concentrations for recovery percentage calculations.

2.6. Reference assay

Quantification of cTnI concentrations in spiked plasma samples was validated via reference assay. The cTnI calibrators and the samples pepared from human plasma pool by spiking with 0–250 ng/L I-T-C-complex were divided in two idential aliquots to be assayed with the developed assay and a commercial assay (Siemens Atellica IM hs-TnI) at HUSLAB (Helsinki, Finland). The limit of quantitation of the reference assay was 3 ng/L cTnI.

2.7. Depletion of cTnI from plasma

Depletion of cTnI from the plasma pool was performed by using the capture Mabs 19C7 and 9707 immobilized on a microtiter plate as described for the assay. Pooled plasma was centrifuged as described and incubated on the plate 50 μL/well at room temperature in slow shaking for one hour, followed by collection and pooling. The cTnI concentration measured from the depleted plasma pool was subtracted from the concentration measured from the non-depleted pooled plasma in a cTnI assay (as described) to estimate the amount of inherent cTnI.

2. Results

3.1. The effect of base in PAA-coating to monodispersity and assay performance

The pH in the PAA-coating reaction was adjusted to 9 using either NaOH or 1,8-Diazabicyclo[5.4.0]-7-undecene (DBU). Using DBU in the PAA-coating reaction yielded PAA-UCNPs with narrower size distribution and larger portion of monodispersed particles compared to using NaOH, characterized by gel electrophoresis[18] (Fig. 2a). The analyte specific UCL signals in a cTnI assay were doubled for antibody conjugated UCNPs (Mab-UCNPs) which had been PAA-coated using DBU (Fig. 2b). The LoD (as 3 × SD of zero calibrator) reached 2.7 ng/L with Mab-UCNPs PAA-coated with DBU, being 4.4-fold more sensitive compared to the coating where NaOH was used, with which the LoD was 12.0 ng/L (assayed with 20% calibrator in sample buffer).

3.2. The effect of EDC to sulfo-NHS ratio to monodispersity and binding efficiency

The effect of EDC-to-sulfo-NHS-ratio in bioconjugation of UCNPs was

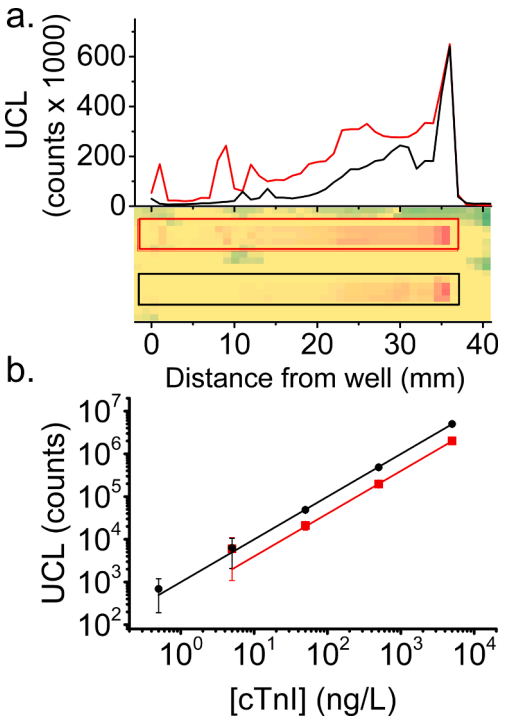

Fig. 2. Comparison of UCNPs with either DBU (black) or NaOH (red) used in the pH-adjustment of PAA-coating reaction. a) UCL intensity graph of agarose gel electrophoresis on PAA-UCNPs, with scanning composite 2D-image of the agarose gel (scanned under 976 nm excitation and measured at 540 nm). b) Calibration curves of corresponding Mab-UCNPs in a cTnI-assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

characterized by using 2.5–30 mM EDC with 30 mM sulfo-NHS in a conjugation reaction. The EDC concentration was inversely proportional to the cluster formation tendency of the Mab-UCNPs throughout the tested EDC-concentrations. The percentage yield of Mab-UCNPs in buffer filtered through 0.1 μm pore size membrane almost doubled when the EDC concentration was decreased from 20 mM to 2.5 mM (Fig. 3a). The yield of filtration after incubation in plasma also increased by about 40% (Fig. 3b). The binding efficiencies of Mab-UCNPs, defined as concentration normalized analyte-bound fractions of the Mab-UCNPs added in the well, were over 5-fold when 2.5 mM EDC was used compared to 30 mM EDC. A clear increase in binding efficiency was seen throughout the series (Fig. 3c).

3.3. Assay performance and cTnI-depletion of health human plasma pool

The effects of assay reagents and protocol to the detection performance are shown in the SI, including different streptavidin brands, the pH of wash buffer after Mab-UCNP incubation, type of anti-cTnI 9707 capture antibody, changes in the relative proportion of the sample volume, the incubation time and the concentration of Mab-UCNP. The calibration curve of the optimized upconversion-based sandwich immunoassay with shown LoB and LoD (based on CLSI-criteria described in the SI) and within- and between-run precisions is shown in Fig. 4. The calibration curve was drawn using zero calibrator subtracted UCL signals and was linear between 0.05 and 500 ng/L cTnI ($y = ax + b$, where $a = 22346 \pm 229$ and $b = 131 \pm 150$, $R^2 = 1.00$). For the linear range of concentrations, the average within-run coefficients of variation were 2.0–4.7% and between-run 4.8–7.2%, except for 500 ng/L, which was 8.8%, all for five runs on five days. The LoB and LoD based on the CLSI-criteria were 0.01 and 0.13 ng/L cTnI, respectively, and the LoD calculated as 3 × SD of zero calibrator for comparison was 0.06 ng/L. The four low analyte level samples used for LoD determination were

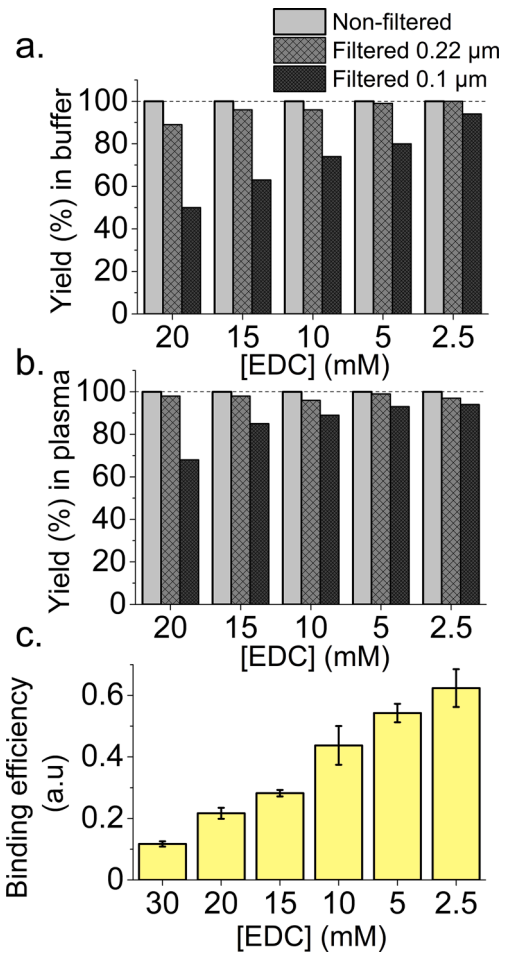

Fig. 3. Mab-UCNP percentage yields from filtration tests of Mab-UCNPs conjugated using 2.5–20 mM EDC, incubated with 20% of either a) 7.5% BSA-TSA, or b) plasma, in sample buffer. c) Binding efficiency of said Mab-UCNPs, including also 30 mM EDC. Error bars represent SD of binding efficiency values.

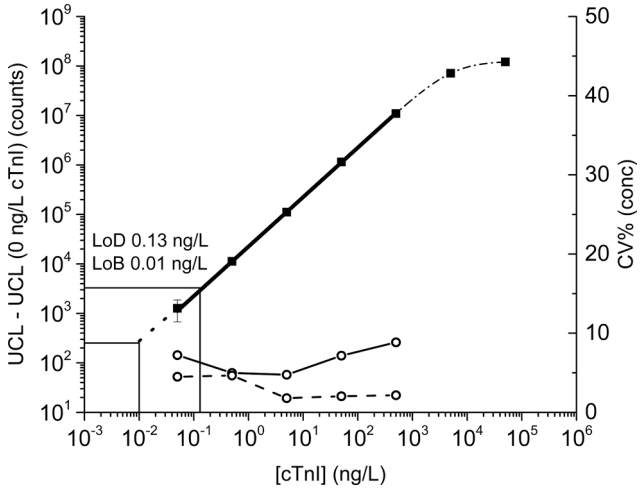

Fig. 4. Calibration curve. The linear range is highlighted as bold black line, and concentrations exceeding it as the dashed and dotted line. Linear range is extrapolated to LoB (dashed line). Error bars represent SD of five repeats in linear range and of one repeat above it. Dashed and solid lines with white spheres represent within- and between-run imprecisions respectively. UCL was measured at 540 nm.

measured with this assay to have inherent cTnI concentrations between 0.24 and 1.34 ng/L (24–134 × LoB). The cTnI recovery percentages from spiked plasma pool were between 91 and 97%, apart from 0.05 and 5 ng/L cTnI-spiked samples, which had 130% and 86% recoveries, respectively. Results of the analysis of the employed calibrators and spiked plasma pool samples with the reference assay are shown in the SI (Table S1).

Healthy human plasma pool was depleted of cTnI via incubation in microtiter plates coated with biotinylated cTnI-capture antibodies, and the UCL signal was measured with the assay. The zero-calibrator subtracted UCL signal of the plasma pool dropped by 83% when plasma was depleted of cTnI and corresponded to concentration decrease of 0.84 ng/L cTnI (Figure S6).

4. Discussion

4.1. Surface chemistry of the reporter

Surface chemistry of the Mab-UCNP reporter was shown in this study to significantly affect the performance of the assay. Replacing NaOH with organic base DBU in the ligand exchange step was shown to increase the proportion of monodisperse Mab-UCNPs. Hypothetically, DMF in the coating medium could react with NaOH[20] yielding formate and dimethylamine, the latter of which would further react with the carboxylic acid groups of PAA, for example leading to reduction of solubility or polymerization. Such pathway would lead to less PAA being available in the reaction. The hypothesis was supported by the formation of a visible pellet when DMF, NaOH and PAA are mixed, but not in the absence of any of the three, or when NaOH was replaced with DBU. Also, when adding dimethylamine directly to a solution of DMF and PAA, a similar pellet was formed (results not shown). The mechanism for the improved behaviour of the conjugate with DBU was, however, not studied further.

Improving the monodispersity of the reporter by changing the base was shown to correlate with improved signal level in the assay. This may be due to increased proportion of discrete reactive units in the same mass of Mab-UCNPs (i.e. higher molar concentration) and reduced steric hindrance in detection of the surface bound analyte due the absence of large UCNP-clusters. This hypothesis was also supported by the increased binding efficiency with further improved monodispersity of the reporter after optimization of EDC to sulfo-NHS ratio.

4.2. Assay optimization

After optimizing the surface chemistry of the UCNP reporter, the effect of other assay components and the protocol were studied to further improve sensitivity of cTnI detection. The brand of streptavidin used to coat the assay plates was shown to have an immense effect on the assay performance. The differences between UCL signal-to-background-ratios of calibrators were 3.5-fold between the most and least suitable streptavidin type (Figure S2a). Using the best streptavidin also reduced non-specific binding in plasma, which can be seen as reduced signal level in non-spiked plasma (Figure S2b).

Higher pH of wash buffer after Mab-UCNP incubation decreased the level of non-specific binding leading to improved UCL signal to background ratios up to pH 10.25, followed by a substantial loss in analyte specific signal at pH 10.75 (Figure S3). This indicates that some of the non-specific binding is charge-related. The negative charge of PAA-coated UCNPs may efficiently bind to positively charged amino-acid residues on the plate surface but in pH 10.25, most of the residues have a negative charge.[21] The loss of analyte specific signal at higher pH is possibly due to deterioration of interactions caused by protein denaturation.[22]

By changing the type of anti-cTnI 9707 binder from Fab to the entire antibody, higher analyte specific UCL signals were measured in both buffer and plasma (Figure S4), in contrast to previous research, where using the Fab-version reduced the level of matrix-related interferences in a cTnI assay.[23] After optimization of other features, the proportion of sample (calibrator or spiked plasma pool) in sample buffer could be increased from previous 20%[310] to 80% for maximal analyte specific signals (Figure S4), showing successful reduction of matrix related interferences in this research.

The analyte binding kinetics of the Mab-UCNPs were studied by altering the length of detection incubation (Figure S5a). From 60 min onward, the change rate of analyte specific UCL signal decelerated to that of non-specific binding, and no change in the signal-to-background ratio was observed between 60 and 120 min. Half of the maximum signal was already achieved in 15 min. Using an incubation time longer than 15 min would not improve the performance of the assay significantly enough when considering the increase in assay duration, and thus it was chosen as the optimal incubation time of Mab-UCNP-tracer in the assay.

In previously published UCL assays, the binding kinetics of Mab-UCNPs with time has been rather similar.[310] However, in both the previous assays the growth-rate of analyte-specific UCL signal is slowed down and overcome by the growth-rate of the background signal after either 15,[3] or 30[10] minutes, which can be seen as a decrease of signal-to-background ratio. Thus, the non-specific binding of the Mab-UCNPs is significantly less pronounced in the assay presented here, with the optimized surface chemistry and assay conditions.

The optimal reporter concentration for the UCL assay was defined as the concentration of Mab-UCNPs, with which the UCL signal-to-background-ratio in buffer was as high as possible, while the cTnI recovery from plasma was the closest to 100%. Using 200 ng/well Mab-UCNPs filled the criteria with recovery of 91% without a significant drop in signal-to-background-ratio compared to the concentration with the highest signal-to-background-ratio (Figure S5b).

4.3. Assay performance and reference assay

In addition to the remarkably low LoD obtained according to CLSI guidelines, extensive evidence was provided for the hypothesis that the assay can reliably detect and quantify cTnI concentrations below the range of healthy human population. The cTnI concentrations of the four low analyte level samples used for LoD determination fell in the range of normal cTnI levels in healthy human population, according to a study conducted with Singulex Clarity cTnI-assay. [24] However, as cTnI is lacking international standardization, reference values should be determined separately for each method.[25] Alternatively, healthy human plasma pool was depleted of cTnI and a clearly measurable decrease in plasma concentration upon the cTnI depletion was observed with the developed assay. In addition to supporting the hypothesis, this result also confirmed that the assay background measured for the plasma pool was principally due to the small concentration of cTnI present and not the sample-associated non-specific binding.

Duplicates of the employed calibrators and spiked plasma pool samples were further assayed using Siemens Atellica IM hs-TnI (limit of quantification 3 ng/L). The results showed excellent agreement between the two assays in all concentrations above 3 ng/L. Since the UCL-assay exhibited flawless linearity, the results supported the validity of the reported cTnI-concentrations also below 3 ng/L down to the LoD. The reference assay also confirmed that the concentration of the plasma pool was less than 3 ng/L.

The excellent correspondence between the two assays can be partly attributed to a similar setup and antibody configuration between the two assays. The Siemens Atellica is also a three-site sandwich immunoassay, with two out of three detected epitopes overlapping with the ones detected in the UCL assay (antibodies in Siemens Atellica detect cTnI amino acid regions 41–50, 171–190 and 29–34 [9], while the antibodies used in the UCL assay detect regions 41–49, 171–190 [26] and 190–195 [27]).

5. Conclusion

In this research we optimized the surface chemistry of UCNP-antibody conjugates, as well as the reagents and assay protocol for highly sensitive detection of cTnI. We succeeded to reduce the cluster formation tendency of the UCNP-conjugates and their non-specific binding in the assay, resulting in unprecedented sensitivity in cTnI detection. The assay was able to reliably quantify the cTnI levels of the plasma pool of healthy volunteers, and the LoD achieved (0.13 ng/L calculated according to CLSI guidelines) is over 10-fold more sensitive than reported for the majority of most commercial hs-cTnI assays, and has not been reached before in an analog sandwich immunoassay format with other reporter systems. Currently, this LoD is only surpassed by Singulex Clarity with LoD of 0.08 ng/L, which uses digital detection on a highly specialized assay platform. Although comparability between cTnI assays is uncertain due to the lack of international standardization, the results were fully comparable with the Siemens Atellica hs-TnI. The outstanding sensitivity achieved using the UCL intensity readout and standard 96-well microtitration plate platform demonstrates the unique potential of UCNPs as reporters in ultra-sensitive biomolecule detection.

CRediT authorship contribution statement

Kirsti Raiko: Conceptualization, Formal analysis, Investigation, Writing – original draft, Visualization. **Annika Lyytikäinen:** Formal analysis, Investigation, Writing – review & editing, Visualization. **Miikka Ekman:** Formal analysis, Investigation, Writing – review & editing. **Aleksi Nokelainen:** Methodology, Writing – review & editing. **Satu Lahtinen:** Conceptualization, Methodology, Writing – review & editing. **Tero Soukka:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

TEM-imaging and size determination of UCNPs, effect of streptavidin type in plate coating, effect of wash buffer pH after conjugation incubation in assay, analyte-binding kinetics of label conjugate and label concentration in assay, effect of cTnI depletion of healthy human plasma pool, and results of comparison with Siemens Atellica IM hs-cTnI (PDF). Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2021.10.023.

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