Supporting Information

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Effect of Particle Size and Surface Chemistry of Photon-Upconversion Nanoparticles on Analog and Digital Immunoassays for Cardiac Troponin

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1 Chemicals and Reagents

The following chemicals were obtained from Merck / Sigma-Aldrich (St. Louis, MO, USA):

- anhydrous $N,N$-dimethylformamide (DMF):
  - biotinylated bovine serum albumin (BSA-biotin)
  - Na$_2$B$_4$O$_7$
  - bovine γ-globulin (BGG)
  - copper(II) sulfate pentahydrate
  - D-sorbitol
  - D-(+)-trehalose
  - heparin sodium salt from procine intestinal mucosa ($\geq 180$ USP units/mg)
  - nitrosyl tetrafluoroborate (NOBF$_4$)
  - $N$-(3-dimethylaminopropyl)-$N'$-ethylcarbodiimide (EDC)
  - $N$-hydroxysulfo succinimide sodium salt (sulfo-NHS)
  - NHS-LC-biotin
  - NHS-dPEG$_8$-azide
  - 2-(N-morpholino)ethanesulfonic acid monohydrate (MES)
  - poly(acrylic acid) (PAA, $M_w$ 2000 Da, 50% (w/v) solution in H$_2$O)
  - poly(acrylic acid) sodium salt (PAA, $M_w$ 1200 Da, 45% (w/v) in H$_2$O)
  - sodium borate decahydrate
  - Tris(hydroxymethyl)aminomethane (Tris)
  - Tween 20
  - Tween 85 (with NaN$_3$
  - YCl$_3 \times 6$ H$_2$O (99.99%)
  - Y$_2$O$_3$ (99.99%)
  - YbCl$_3 \times 6$ H$_2$O (99.998%)
  - Yb$_2$O$_3$ (99.99%)
  - ErCl$_3 \times 6$ H$_2$O (99.99%)
  - Er$_2$O$_3$ (99.99%)
  - NH$_4$F (>98%)
  - octadec-1-ene (technical grade, 90%)
  - oleic acid (technical grade, 90%)
  - trifluoroacetic acid (99%)
The following chemicals were obtained from Penta (Prague, Czech Republic):
  - cyclohexane (p.a.)
  - methanol (p.a.)
  - NaHCO₃
  - NaOH (p.a.)
  - propan-2-ol (p.a.)

Further chemicals:
  - 2-amino-N,N-dimethylacetamide (ADMA, Combi-Blocks, San Diego, CA, USA)
  - bovine casein (Calbiochem, La Jolla, CA, USA)
  - bovine serum albumin (BSA, Bioreba, Reinach, Switzerland)
  - fat-free bovine milk powder (Valio, Helsinki, Finland)
  - α-N-hydroxysuccinimide-ω-alkyne poly(ethylene glycol) (NHS-PEG-Alkyne, \( M_w \) 3000, Iris Biotech, Marktredwitz, Germany)
  - IgG, mouse, purified on protein A (9–13 mg/mL, 500 mg NaN₃, Meridian Life Science, Memphis, TE, USA). Denatured mouse IgG was prepared by heat denaturation at 63 °C for 30 min.
  - poly(vinyl alcohol) (PVA, \( M_w \) 6000 Da, Polysciences, Warrington, PA, USA)
  - streptavidin (Thermo Fisher Scientific)
  - streptavidin-azide (7 Bioscience, Neuenburg, Germany)
  - SuperBlock (TBS) blocking buffer (Thermo Fisher Scientific)
  - wash solution and colourless buffer solution (Kaivogen, Turku, Finland)

2 Biotinylation of Antibodies mAb560 and mAb625

The biotinylation reaction was carried out at room temperature. First, 30 µL of mAb560 or mAb625 (3.18 mg/mL in PBS) were mixed with 1.15 µL of NHS-LC-biotin (5 mg/mL in dry DMF). After 10 min under shaking, another 1.15 µL of NHS-LC-biotin was added and the mixture shaken for 2 h. The biotinylated antibodies were purified via 6 times centrifugation (14,000 g, 20 min) using Amicon ultra centrifugal filters (\( M_w \) cut-off 100 kDa, Merck, Darmstadt, Germany), transferred to PBS, and stored at 4 °C in the concentration of 1 mg/mL.
3 Synthesis of UCNPs for the Preparation of UCNP-PEG-SA Labels

3.1 Synthesis of Seed UCNPs

YCl₃ × 6 H₂O (1165 mg, 4.8 mmol), YbCl₃ × 6 H₂O (335 mg, 0.864 mmol) and ErCl₃ × 6 H₂O (36.8 mg, 0.096 mmol) were dissolved in methanol (30 mL) and added into a 250-mL three-neck round-bottom flask containing oleic acid (32.3 g, 36 mL) and 1-octadecene (19.7 g, 84 mL). The solution was heated to 170 °C under an N₂ atmosphere for a time long enough to remove all volatile liquids (approximately 60 min) and then the temperature was decreased to 50°C. The protective atmosphere was disconnected, and the solution of NH₄F (711 mg, 19.2 mmol) and NaOH (480 mg, 12 mmol) in methanol (30 mL) was added to the intensely stirred solution. The N₂ atmosphere was reconnected, and the solution was stirred for 30 min. The temperature was carefully increased up to 150 °C, avoiding extensive boiling to ensure the evaporation of methanol. After that, the solution was rapidly heated at the rate of ~10 °C per min. At 290 °C, the heating was carefully regulated to 300 °C within one or two minutes. The flask was kept under N₂ flow at 300 °C for 90 min. The fluctuation of temperature was ± 4 °C during this time. Finally, the flask was cooled down to room temperature. The resulting nanoparticles were precipitated by adding propan-2-ol (240 mL) and collected by centrifugation (1,000 g, 10 min). The pellet was washed with methanol (90 mL), centrifuged (1,000 g, 10 min), and dispersed in cyclohexane (20 mL). By adding methanol (100 mL), the nanoparticles precipitated rapidly without the need for centrifugation. The precipitate was dispersed in cyclohexane (30 mL) and slowly centrifuged (50 g, 20 min) to separate coarse particles from the final product.

3.2 UCNP Growth

Under reflux, Y₂O₃ (1355 mg, 6.00 mmol), Yb₂O₃ (532 mg, 1.35 mmol) and Er₂O₃ (57.9 mg, 0.15 mmol) were dissolved in trifluoroacetic acid (12 mL) and water (12 mL) in a 250 mL three-necked flask. When dissolved, NaHCO₃ (1260 mg, 15.00 mmol) was added releasing CO₂ bubbles and dissolving rapidly, resulting in a clear solution. After removing the condenser, excessive trifluoroacetic acid and water were evaporated by heating at 110°C in a fume hood (overnight). The resulting white powder of trifluoroacetates was dissolved in oleic acid (45 mL, 40.3 g) and octadec-1-ene (45 mL, 35.5 g). This solution was diluted by 30 mL of methanol. The methanol together with oxygen and water were removed by heating at 110°C under the N₂ atmosphere for 20 min, resulting in a precursor solution. The precursor solution was enclosed in the flask by silicon septa and kept under an inert atmosphere. To decrease the viscosity, the precursor solution was kept at an elevated temperature (~50 °C), which facilitated its injection.
into the hot reaction mixture. The concentration of Re(CF₃CO₂)₃ in the precursor solution was 0.17 mmol mL⁻¹ (Re for Y, Yb, Er in molar percentages 80%, 18% and 2.0%, respectively.

The nanoparticles were grown by gradually adding the precursor solution to the solution of seed nanoparticles. The dispersion of seed nanoparticles (205 mg) in cyclohexane was mixed with oleic acid (5.5 mL, 4.9 g), octadec-1-ene (17 mL, 13.4 g), and 20 mL of methanol in a 100-mL three-necked flask. The mixture was heated at 150 °C for ~30 min under the N₂ atmosphere to remove oxygen and water. Then, the temperature was rapidly increased to 300 °C. Without decreasing the temperature, a calculated amount of precursor solution was repeatedly injected by a syringe with a long needle (120 mm length); the 9 subsequent addition volumes corresponded to 3.5, 4.0, 4.6, 5.6, 6.2, 7.1, 8.3, 9.6, and 10.1 mL; the time interval between injections was 10 min. The volume of the reaction mixture eventually reached the capacity of the 100-mL flask. Therefore, the reaction mixture was transferred to a 250-mL three-necked flask together with 30 mL of methanol. Under an inert N₂ atmosphere, the oxygen, methanol, and water were removed by heating at 150 °C for ~30 min. The temperature was rapidly increased to 300 °C, and the injection procedure was repeated. After the last injection, the temperature was kept at 300 °C for 10 min, eventually preparing nanoparticles of the desired size (reaction mixture volume ~82 mL), and the synthesis continued without decreasing the temperature by growing the inert shell of NaYF₄.

3.3 Inert Shell Growth

Under reflux, Y₂O₃ (1694 mg, 7.50 mmol) was dissolved in trifluoroacetic acid (12 mL) and water (12 mL) in a 250-mL three-necked flask. When dissolved, NaHCO₃ (1260 mg, 15.00 mmol) was added releasing CO₂ bubbles and dissolving rapidly to a clear solution. After removing the condenser, excessive trifluoroacetic acid and water were evaporated by heating at 110 °C in a fume hood (overnight). The resulting white powder of trifluoroacetates was dissolved in oleic acid (45 mL, 40.3 g) and octadec-1-ene (45 mL, 35.5 g). This solution was diluted by 30 mL of methanol. The methanol together with oxygen and water were removed by heating at 110 °C under an inert N₂ atmosphere for 20 min resulting in a precursor solution. The precursor solution was enclosed in the flask by silicon septa and kept under an inert atmosphere. To decrease the viscosity, the precursor solution was held at an elevated temperature (~50 °C), which facilitated its injection into the hot reaction mixture. The concentration of Y(CF₃CO₂)₃ in the precursor solution was 0.17 mmol mL⁻¹.
The shell was grown by gradually adding the precursor solution to the hot solution of grown nanoparticles from the previous step. A calculated amount of precursor solution was repeatedly injected by a syringe with a long needle (120 mm) without decreasing the temperature. The three additions were of 8.0, 9.0, and 10.0 mL; the time interval between the injections was 10 min. After the last injection, the temperature was kept at 300 °C for an additional 10 min, eventually preparing the desired nanoparticles (the volume of the reaction mixture ~95 mL). Finally, the flask was cooled to room temperature. The resulting nanoparticles were precipitated by adding propan-2-ol (190 mL) and collected by centrifugation (1,000 g, 10 min). The pellet was washed with methanol (109 mL), centrifuged (1,000 g, 10 min), and dispersed in cyclohexane (60 mL). After the last precipitation by methanol, the nanoparticles were dispersed in cyclohexane and slowly centrifuged (50 g, 20 min) to separate coarse particles from the final product.

4 Preparation of Click-Reactive Streptavidin

For the preparation of click-reactive streptavidin-azide, 31.25 µL of NHS-dPEGs-azide (200 mM, Sigma-Aldrich, St. Louis, MO, USA) in DMF were added to 150 µL of streptavidin (4.0 mg/mL) in DMF. The 187.5 µL of phosphate buffer (50 mM NaH₂PO₄/Na₂HPO₄, pH 7.4) were added, the reaction mixture was incubated for 2 h at room temperature, and then quenched by the addition of Tris-HCl (50 mM, pH 7.4). The streptavidin-azide was purified via 6 times centrifugation (14,000 g, 20 min) using Amicon ultra centrifugal filters (Mw cut-off 10 kDa, Merck, Darmstadt, Germany), transferred to PBS, and stored at 4 °C in the concentration of 1 mg/mL.

5 Characterization of UCNPs and UCNP Conjugates

5.1 Transmission Electron Microscopy (TEM)

Oleic acid-capped UCNPs (NaYF₄:Yb³⁺, Er³⁺) in cyclohexane were diluted and dispensed on carbon-coated copper grids. The excess dispersion was removed and the grids were allowed to dry on air. TEM images were recorded either on a Titan Themis (FEI, Czech Republic) or a JEM-1400 Plus (JEOL, Massachusetts, USA).
Figure S1. TEM-images of NaYF₄: Yb³⁺, Er³⁺-UCNPs of sizes (a) 63 nm (used for the SA-PEG-UCNP conjugates), and (b) 40 nm, (c) 48 nm, (d) 56 nm, (e) 64 nm, (f) 80 nm (used for the mAb-PAA-UCNP conjugates).
5.2 Dynamic Light Scattering (DLS)

The hydrodynamic diameters of the UCNPs and their conjugates were determined by DLS using a Zetasizer Nano (Malvern, UK). A dispersion of 187 µg/mL for Er-doped oleic acid capped UCNPs (in cyclohexane) and 325 µg/mL (in 50 mM Tris) for their bioconjugates were used for the characterization.

![DLS particle size distributions](image)

**Figure S2.** DLS particle size distributions (by intensity) of (a) SA-PEG-UCNP with oleic acid coating (in cyclohexane) and after conjugation with streptavidin (in 25 mM Tris), and (b) mAb-PAA-UCNP conjugates (in water). The size distributions are summarized in Table S1.
5.3 Upconversion Emission Spectra

Figure S3. Spectrum of UCNPs (NaYF₄:Yb³⁺,Er³⁺, 63 nm in diameter) under 980-nm excitation.

6 Optimization of the Troponin Detection System

Figure S4. Comparison of label combinations for the detection of cTnI in BSA/BGG buffer. (a) ULISA configuration with mAb-coated microtiter plates and SA-PEG-UCNP labels (63 nm in diameter) in combination with biotinylated detection antibodies. (b) ULISA configuration with streptavidin-coated microtiter plates and mAb-PAA-UCNP detection labels (25 nm in diameter). In further experiments, UCNPs of 40–80 nm in diameter were used to enable not only the optimization of the analog, but also of the digital readout.
Figure S5. Digital ULISA of troponin dilutions in buffer. (a–d) mAb-PAA-UCNP (48 nm in diameter). (e–h) mAb-PAA-UCNP (64 nm in diameter). (i–l) mAb-PAA-UCNP (80 nm in diameter). (m–p) SA-PEG-UCNP (63 nm in diameter). Wide-field upconversion microscopy images and brightness distribution histograms of 500 diffraction-limited spots. Scale bar: 10 µm.
Figure S6. Digital ULISA of troponin dilutions in plasma. (a–d) mAb-PAA-UCNP (48 nm in diameter). (e–h) mAb-PAA-UCNP (64 nm in diameter). (i–l) mAb-PAA-UCNP (80 nm in diameter). (m–p) SA-PEG-UCNP (63 nm in diameter). Wide-field upconversion microscopy images and brightness distribution histograms of 500 diffraction-limited spots. Scale bar: 10 µm.
**Table S1. Homogeneity of UCNP labels**

| Type of label/assay | Size (nm) | Label brightness in buffer | Label brightness in plasma | Hydrodynamic size distribution |
|---------------------|-----------|---------------------------|---------------------------|-------------------------------|
| mAb-PAA-UCNP        | 40        | n.d.                      | n.d.                      | 40                            |
|                     | 48        | 15 ± 12                   | 34 ± 17                   | 41                            |
|                     | 56        | 49 ± 30                   | 63 ± 39                   | 37                            |
|                     | 64        | 32 ± 14                   | 55 ± 32                   | 42                            |
|                     | 80        | 121 ± 58                  | 114 ± 56                  | 29                            |
| SA-PEG-UCNP         | 63        | 12 ± 7                    | 18 ± 7                    | 32                            |

a Average UCNP diameter determined by TEM (Figure S1).

b Mean (μ) luminescence intensity ± standard deviation (σ) of diffraction-limited spots based on LogNormal fit (Figures S5–S6).

c Calculated as $100\sqrt{\text{PDI}}$ (Figure S2).

d Not determinable because smaller UCNPs are not bright enough for single nanoparticle detection.

### 8 Digital Readout Using SA-PEG-UCNP Labels

**Figure S7.** Calibration curves of the digital ULISA using biotinylated mAb 560cc and SA-PEG-UCNP labels. cTnI was serially diluted in either BSA/BGG buffer (LOD: 19.0 pg/mL) or 20% plasma (LOD: 31.0 pg/mL) in BSA/BGG buffer. The error bars show the standard deviation of three replicate measurements; the hatched lines indicate the LODs.