Electronic Supplementary Material (ESI) for Analytical Methods.

Highly sensitive detection of C-reactive protein using a novel dissolution approach in a dye-doped silica nanoparticle-based fluorescence immunoassay

Materials, methods and instrumentation.

Materials

Hydrophobic and hydrophilic Cyanine5 analogues of Cy5® were purchased from Lumiprobe GmbH. Molecular sieve 3A (4-8 mesh), dimethyl sulfoxide (>99.9 %), Ethanol (99.5 %), Aminopropyltrimethoxysilane (APTMS, 99 %), bis [3-(triethoxysilyl) propyl] amine (95 %), polyoxyethylene (5) nonylphenylether (NP5), dioctyl sulfosuccinate, sodium salt (AOT, 98 %), cyclohexane (anhydrous, 99.5 %), tetraethylorthosilicate (TEOS, 99.999 %), N-hydroxysulfosuccinimide sodium salt (NHS, > 98.5 %), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 98+%), phosphate buffered saline tablets (one tablet dissolved in 200 mL of deionised water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C), sodium carbonate (>99%), sodium bicarbonate (>99,7%), 2-(N-Morpholino)ethanesulfonic acid hydrate (MES hydrate, > 99.5 %), Polyoxyethylene (20) sorbitan monolaurate (Tween 20®), D-(+)-trehalose dehydrate (>99 %), ammonium hydroxide solution (28 (w/w)), sodium dodecyl sulfate (SDS, >99%), albumin from bovine serum (BSA, freeze dried powder, ≥96%), anti-hIgG (Fab Specific) polyclonal antibody produced in goat, and IgG from human serum (>95 %) were all purchased from Sigma Aldrich. The antibody linker, 8-arm PEG succinimidyl NHS ester (10000 Da) and the block, amine functionalized PEG (5000 Da) were both purchased from Nanocs Inc. Human C-reactive protein was purchased from Millipore. The capture and detection antibodies, purchased from Hytest Ltd, were monoclonal mouse anti-human C-reactive protein C2 and C6 clones, respectively. The free dye used as label in immunoassay for the detection of C-Reactive protein was, DY-647P1-NHS-ester, purchased from Dyomics GmbH. Deionised water (<18 MΩ) was obtained from a Milli-Q system from Millipore, Ireland.

Methods and Calculations

Synthesis of Cyanine5 NPs

Four sets of NPs were prepared containing initial dye amounts of 2, 4, 6, and 8 % Cyanine5 (w/w) following a previously published protocol.[1]

The first step was to conjugate hydrophobic Cyanine5 to APTMS using the following protocol. Cyclohexane (3 mL) and NP5 (1 mL) were mixed and dried over 3A molecular sieve beads for 24 hours. To a dried glass vial placed in a nitrogen glove box were added Cyanine5 dye (4.6 mg), cyclohexane/NP5 mix (1.5 mL) and APTMS (6.49 μL). The contents were stirred for four hours to complete conjugation of dye to organosilane.

In the second step a microemulsion was prepared to synthesise dye doped NPs using the following protocol. In four plastic bottles were added 260 mg of AOT followed by 9.9, 9.8 9.7, 9.6 and 9.5 mL of cyclohexane and 194, 157, 118, and 77 μL of NP5, respectively. Following this 70 μL of H_2O was added to each and 10 minutes later 144, 294, 450, and 613 μL of conjugate

mixture added, respectively. Immediately following this, TEOS (80 μL), and 20 minutes later, ammonium hydroxide solution (60 μL, 28 (w/w)) were added to each with stirring for 24 hours. The microemulsions were broken with the addition of ethanol (30 mL). Finally, the nanoparticles were purified by centrifugation in ethanol (3x, 24000 RCF, 8 minutes). The actual dye loadings determined from UV-vis absorption experiments of known NP concentrations were 0.38, 0.71, 0.81 and 1.16 % (w/w) Cyanine5. The total NP yields were 14, 14, 18 and 19 mg with corresponding yields of 64, 62, 72, and 74 % respectively versus maximum theoretical yields.

Synthesis of silica shells.

To investigate the effect of shell thickness on rate of dissolution NPs were coated with five different shell thicknesses using a modification of a previously published protocol.[2] Five aliquots, each containing 0.71 % (w/w) Cyanine5 NPs (5.2 mg), ethanol (3.25 mL) and deionised water (680 μL) were prepared. Following this 2.5, 10, 15, 20 and 100 μL of TEOS and ammonium hydroxide (100 μL) were added to each aliquot, respectively, with stirring for 24 hours. Finally, the nanoparticles were purified by centrifugation in ethanol (6 mL, 3x, 24000 RFC, 8 minutes).

Calculation of shell thicknesses.

Shell thickness were calculated from the amount of TEOS added to each sample using a similar approach to that of Liz-Marzan *et al*.[3] The first part is to calculate the molar volume of silica, $\bar{V}_{SiO_2}$. By convention the molar volume is defined as the molar mass divided by the density. However, Masalov *et al.* proved that the density of silica in nanoparticles is not constant.[4] According to Masalov *et al.* silica NP cores have a density of 1.6 cm^{-3} from the close packing of primary particles. However, any residual TEOS at concentrations below the nucleation saturation concentration coat silica NPs at a density of 2.22 g cm^{-3}. In this work we assume a density of 2.22 g cm^{-3} for the shell, which can be regarded as an upper bound for the density as it may be possible that a small number of primary particles will also form during synthesis which would lower this density. Therefore, the molar volume in this work is equal to 27.39 cm^3.

The volume per nanoparticle, V_{NP} is then calculated from the following equation,

$$V_{NP} = \frac{\bar{V}_{SiO_2}.mols\ of\ silica\ present\ in\ x\ micro\ litres\ of\ TEOS}{Number\ of\ core\ nanoparticles\ in\ 5.2\ mg}$$

To calculate the number of NPs in 5.2 mg without shell, each NP was assumed to be spherical with an average radius taken from TEM images and a density of 1.6 g cm^{-1}. The number of nanoparticle was then determined from solving the equation for the volume of a sphere.

The radius of the NP plus shell, r_2, is then calculated from the following equation,

$$r_2 = \left(\frac{3V_{NP}}{4\pi} + r_1^3\right)^{\frac{1}{3}}$$

where r_1 is the radius of the NP without shell in centimetres. The thickness of the shell in nanometres is therefore equal to $(r_2-r_1)x10^7$. To validate this equation experimentally TEM images were taken of 1 % (w/w) Cyanine5 NPs as-prepared, plus 10 μL and plus 20 μL of TEOS, respectively (see figure S1). In addition, the four NPs samples with 0.38, 0.71, 0.81 and 1.16 % (w/w) Cyanine5 plus 200 μL of TEOS were also imaged (see figure S2).

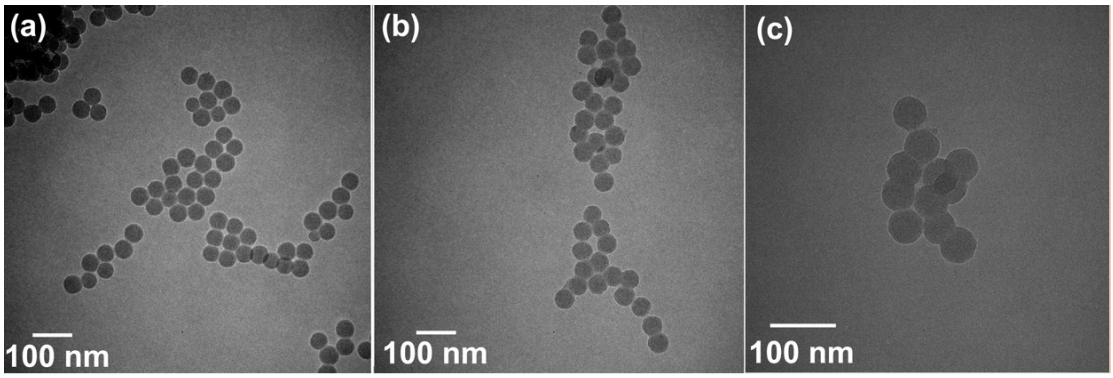

Figure S1) Change in shell thickness with addition of TEOS for as-prepared 1 % (w/w) Cyanine5 NPs (a). Images (b) and (c) show these NPs plus 10 and 20 μL of TEOS, respectively.

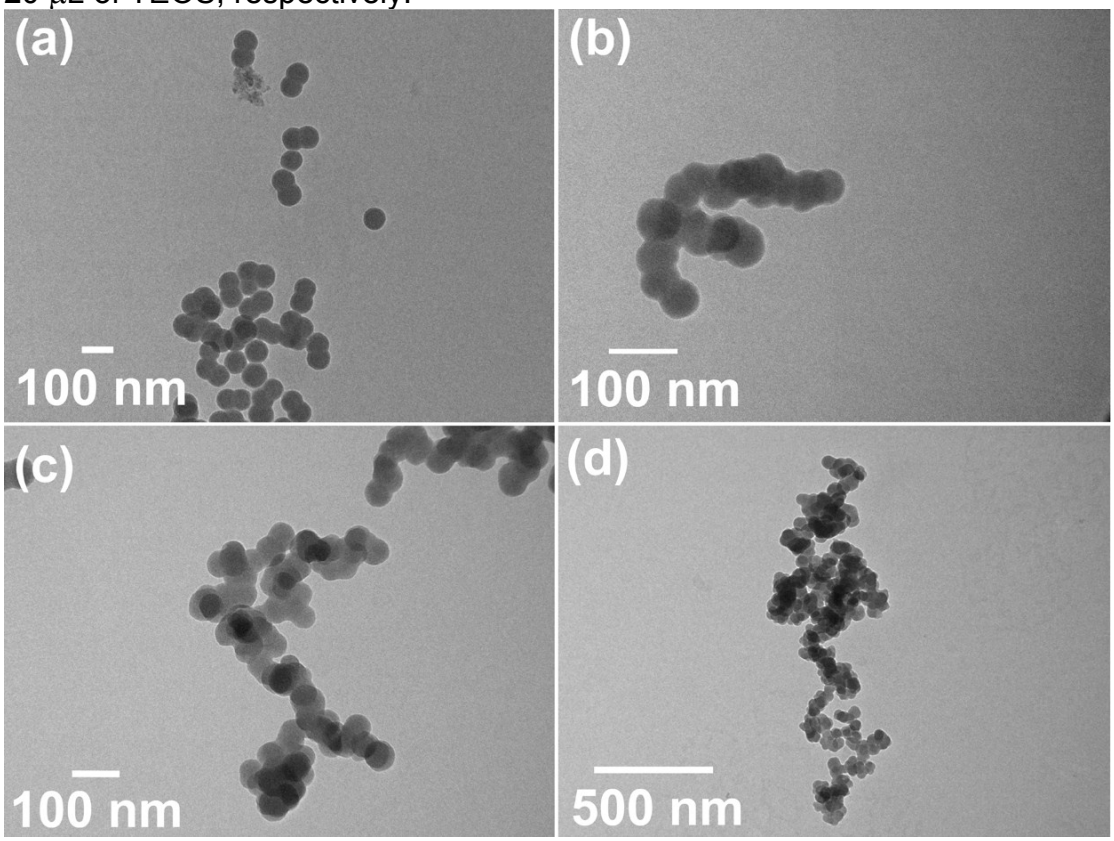

Figure S2) Change in shell thickness with addition of 200 □L TEOS for NPs with 0.38 (a), 0.71 (b), 0.81 (c), and 1.16 (d) % (w/w) Cyanine 5 dye loadings, respectively.

A summary of the experimental results compared to results from TEM is shown in table S1.

Core Diameter (nm)	TEOS (μL)	Core plus shell exp. Diameter (nm)	Core plus shell exp. Shell thickness (nm)	Core plus shell calc. Diameter (nm)	Core plus shell calc. Shell thickness (nm)
45.6 +/- 1.8	0	0	0	0	0
45.6 +/- 1.8	10	50.8 +/- 1.9	2.6	50.7	2.5
45.6 +/- 1.8	20	53 +/- 2.9	3.7	54.9	4.7
39 +/- 2 (a)	200	74.4 +/- 5	17.7	79.5	20.2
35.5 +/- 4 (b)	200	69 +/- 6	16.8	72.4	18.4
29.9 +/- 4 (c)	200	58.7 +/- 5	15.4	56.9	14.5
24.1 +/- 5 (d)	200	51.5 +/- 3.5	13.7	49.1	12.5

Table S1) Comparison between experiment and calculated results for core and core plus shell diameters on addition of known volumes of TEOS to Cyanine5 NPs. The first three rows relate to TEM images in figure S1. The final four rows correspond to images in TEM images in figure S2.

We can see that there is good agreement within statistical error for all experimental data and theoretical predictions for shell thicknesses. This calculation was therefore considered a reasonable method to determine shell thicknesses for 0.71 % (w/w) Cyanine5 NPs used in the kinetic study in the main manuscript. In this case, NPs with TEOS additions of 2.5, 10, 15, 20, and 200 had shell thicknesses of 0.54, 1.98, 2.84, 3.63, and 18.4 nm, respectively. For a description of the fluorescence measurement protocol on samples with difference shell thicknesses see **Kinetic Nanoparticle Dissolution Amplification Fluorescence Analysis.**

Antibody bioconjugation to Cyanine5 nanoparticles.

The first step in bioconjugation is to activate shell coated NPs through attachment of a chemically stable amine group. In brief, a solution of NPs (6 mg) dissolved in ethanol (950 μL) and deionized water (50 μL) was reacted with 3-triethoxysilylpropylamine (20 μL) with stirring for 3 hours. The NPs were then centrifuged three times in ethanol (24000 RCF, 7 minutes). A full characterisation of silica NPs before and after bioconjugation including infrared spectra is provided by Keller *et al*.[5]

The second step is to attach a stable multivalent PEGylated linker that will react with an antibody. In brief, NPs (2 mg) were mixed with a carboxyl functionalized 8-arm PEG linker (1 mg) in MES buffer (1.5 mL, pH = 4.7, 0.1 M) containing EDC (50 mM) for 24 hours. PEGylated NPs were centrifuged three times in ethanol (24000 RCF, 7 minutes).

The third step is the attachment of an antibody to the PEGylated NP. In brief, 2 mg of carboxyl terminated PEG NPs were activated in MES buffer (1.5 mL, pH 4.7, 0.1M) containing EDC (50 mM) and sulfo-NHS (12.5 mM) for fifteen minutes. NPs were centrifuged once (24000 RCF, 7 minutes) and then dissolved into MES buffer (1.5 mL MES buffer, pH = 7.2, 0.1M). To this was added 214 μg of antibody. For application in C-reactive protein assays the NPs were reacted with monoclonal mouse anti-human C-reactive protein C6 antibody. For application in human IgG assays the NPs were reacted with fab specific polyclonal anti-human IgG. After 24 hours' reaction time the nanoparticles were centrifuged three times in PBS (24000 RCF, 7 minutes)

and stored at 0.5 mg / mL in PBS, containing 1 % (w/v) BSA, 0.05 % (v/v) Tween 20® and 0.01 % (v/v) sodium azide. For application in freeze dried assay anti-human CRP coated NPs were spun down and suspended at 0.5 mg / mL in PBS, containing 1 % (w/v) BSA, 0.05 % (v/v) Tween 20®, 4 % D-(+)-trehalose dehydrate and 0.01 % (v/v) sodium azide. Each solution was then aliquoted into 100 μL portions and immersed into liquid nitrogen for 20 seconds. The aliquots were then placed onto an Edmund freeze-drier and evacuated overnight. Each aliquot was then stored at -20 °C until analysis.

In experiments where additional blocks were covalently attached to NPs in an attempt to reduce non-specific binding and subsequently improve assay performance the following protocols were used. For experiments where BSA was added, after the original 24 hours' reaction time in contact with antibody the NPs were spun down and immediately suspended in 1.5 mL of MES buffer (1.5 mL MES buffer, pH = 7.2, 0.1M) containing 0.5 % (w/v) BSA and rotated for a further 24 hours. Following this the NPs were centrifuged three times in PBS (24000 RCF, 7 minutes) and stored at 0.5 mg / mL in PBS, containing 1 % (w/v) BSA, 0.05 % (v/v) Tween 20® and 0.01 % (v/v) sodium azide. For experiments where amine functionalized PEG (5000 Da) was added the same protocol was used except 2 mg of PEG was added in place of BSA.

Free Dye labelling of anti-hIgG

As purchased anti-hIgG in PBS was buffer exchanged with sodium bicarbonate buffer (0.05 M, pH 8.5) using Zeba desalting columns. The antibodies (0.5 mg /mL) were mixed with a solution of Sulfo-Cyanine5-NHS-ester dissolved in DMSO (10 mg /mL) at a molar ratio of 50 dye molecules to 1 antibody for 1 hour. The antibody dye conjugates were purified and buffer exchanged with PBS and stored at -20 °C. The dye to protein ratio was determined to be 3.

Free Dye labelling of anti-CRP antibody

The C6 detection antibody was labelled with DY-647P1-NHS-ester. The C6 antibody was placed in sodium carbonate buffer (0.1 M, pH 8.5) and 20 molar equivalents of the DY-647P1-NHS-ester previously dissolved in DMSO was added. The solution was agitated for 2 hours at room temperature. The DY647-C6 conjugate was purified using a NAP-5 column (GE Healthcare) eluting with PBS pH 7.4. The dye to protein ratio was determined to be 8.

Steady state Nanoparticle Dissolution Fluorescence Amplification protocol.

Experiments were performed on 0.38, 0.71, 0.81 and 1.16 % (w/w) Cyanine5 as prepared NPs, respectively. At time zero NPs (0.1 mg / mL) were suspended into dissolution buffer (Carbonate buffer pH = 10.0, 0.1M) containing 0.5 % (w/v) SDS. Addition of SDS was required to maintain solubility of hydrophobic Cyanine5 dye under aqueous conditions. After one-hour incubation at room temperature to complete dissolution, calibration curves were generated from series dilutions. Aliquots of 150 μL were then added to a black Nunc® microtiter plate. Fluorescence readings were then taken on a Tecan Infinite 200 Spectrophotometer using fixed excitation and emission wavelength of 646 and 676 nm, respectively at a gain of 100.

Calculation of NP Dissolution Fluorescence Amplification from the steady state.

The first step in calculating NP amplification was to determine the exact concentration of NPs in 0.1 mg mL^{-1}. To do this each NP was assumed to be spherical with an average radius taken from TEM images. To find the mass of one NP a density of 1.6 g cm^{-1} was used, which was previously calculated by Masalov *et al.*, from the analysis of close packed silica primary particles.[4] The number of nanoparticle in 0.1 mg and Molar concentration were then easily determined using Avogadro's constant.

The second step was to determine the sensitivity of fluorescence which is herein defined as equal to the slope of the change in fluorescence with change in concentration. It should be noted that for all sensitivity measurement the temperature, volume and instrument parameters were kept constant. In the main text is a graph of change in fluorescence with NP concentration obtained for sample 0.71 % Cyanine5 (w/w) NPs, as prepared and after dissolution (Figure 3). The results are compared against a fluorescence calibration curve for free hydrophilic Cyanine5 in water. The next step was to determine the brightness ratio of as-prepared and dissolved NPs versus free Cyanine5 dye using Lian *et al.* method.[6] In this method the brightness ratio is equal to the sensitivity of fluorescence of the nanoparticle divided by the sensitivity of fluorescence of the free dye. Therefore, for as-prepared NPs the brightness ratio is calculated from dividing 10.2 by 1.32, which equals 7.73. On dissolution the brightness ratio increased to 249, which is also equal to the amplification of fluorescence from nanoparticle dissolution versus a free dye.

Calculation of limit of detection.

In this work the limit of detection is defined as equal to five times the standard deviation of the background signal from solvent with no NP added divided by the sensitivity of fluorescence from the calibration curves.

Kinetic Nanoparticle Dissolution Fluorescence Amplification Analysis.

1. Fluorescence Investigation of NPs without added shells.

For this experiment the concentration of NPs was adjusted to 0.02 mg/mL in deionised water. A 75 μL aliquot of NP solution was then added to the well of a black Nunc® microtiter plate and the temperature adjusted to 25 °C. Following this 75 μL of 2X dissolution buffer (0.2M Carbonate buffer, pH 10.0 and 1% SDS) was added to the well. Immediately following this step, sixty repeat fluorescent measurements were performed over a one-hour period to determine the dissolution rate. Fluorescence readings were performed on a Tecan Infinite 200 Spectrophotometer using fixed excitation and emission wavelength of 646 and 676 nm, respectively and a gain of 100. The concentration of NPs was determined using the method described previously.

2. Fluorescence Investigation of NPs with added shells of different thicknesses.

For this experiment the concentration of NPs was adjusted to 0.1 mg/mL in deionised water. The remaining part of this protocol is the same as that described above.

Nanoparticle Dissolution TEM Analysis.
A 1 mL sample of NPs (0.71 % Cyanine5 (w/w), plus a 20 μL TEOS shell and PEGylated) was dispersed into deionised water at 0.5 mg/mL. A sample was then prepared for TEM analysis by dropping a small portion of the NP solution onto a formvar carbon coated copper grid. This was called time zero. Following this, 500 μL of sample was then mixed with 500 μL of 2X dissolution buffer. After 10, 20, 40, 60 minutes and 24 hours, respectively, aliquots of 100 μL were taken, spun down and suspended into ethanol to stop dissolution. Samples were again dropped onto grids for TEM analysis.

Nanoparticle Stability in Phosphate Buffered Saline TEM Analysis .
A 20 μL aliquot of a 1 mL sample of NPs (0.38 % Cyanine5 (w/w), plus a 20 μL TEOS shell and PEGylated) stored in ethanol at 0.5 mg/mL was dropped onto a TEM grid for analysis. This sample was labelled time zero. Following this, the NP was spun down and dispersed into 1 mL of phosphate buffered saline. After 10 minutes, 30 minutes, 2 hours, 24 hours and one week, respectively, aliquots of 100 μL were taken, spun down and suspended into ethanol to stop dissolution. Samples were again dropped onto grids for TEM analysis.

Fluorescence linked Immunoassay protocols:
CRP NP Immunoassay with Dissolution Amplification.
Mouse anti-human CRP monoclonal antibody C2 (10 μg/mL, 50 μL) was added to the wells of a Nunc® MaxiSorp™ 96 well black microtiter plate. The plate was then incubated overnight at 4°C. To prevent non-specific adsorption, 100 μL of 1 % (w/v) BSA and 10 % (v/v) Tween in PBS was added to each well and the plate incubated at 37 °C for 1 hour. To remove any non-adsorbed antibody the plate was then rinsed three times with PBS Tween20® and three times with PBS. Following this, 50 μL aliquots of human CRP were added in a series of dilutions from 50,000 ng / mL to 0 ng / mL to each well and the plate incubated at 37 °C for 1 hour. The rinse cycle was then repeated to remove any non-specifically bound CRP. Finally, 50 μL aliquots of monoclonal mouse anti-human C-reactive protein C6 antibody conjugated NPs at 0.5 mg / mL were added to each well and the plate incubated for a further hour at 37 °C in the dark. The plate was again rinsed three times with PBS Tween20® and three times with PBS. Fluorescence readings were then taken on a Tecan Infinite 200 Spectrophotometer using fixed excitation and emission wavelength of 646 and 676 nm, respectively at gains of 100, 130 and 150. In the final step, 150μL of dissolution buffer (0.1M Carbonate Buffer, pH 10.0, 0.5% SDS) was added to each well and the plate incubated for a further hour at 37 °C. Fluorescence readings were again taken using the specifications described previously.

CRP Free Dye Assay
To compare the NP label with a free dye label, the NP was replaced with DY647-C6 detection antibody and the following optimised protocol used. Mouse anti-human CRP monoclonal antibody C2 (5 μg/mL, 100 μL) was added to the wells of a Nunc Maxisorp 96 well black microtiter plate and incubated at 4 °C overnight. The plate was then rinsed three times with PBS

Tween20® and three times with PBS. To prevent non-specific adsorption, 200 µL of 1 % (w/v) BSA in PBS was added to each well and the plate incubated at 37 °C for 1 hour. The plate was then rinsed again three times with PBS Tween20® and three times with PBS. Following this, 100 µL aliquots of human CRP were added in a series of dilutions in triplicate from 50,000 ng / mL to 0 ng / mL to each well and the plate incubated at 37 °C for 1 hour. The plate was then rinsed again three times with PBS Tween20® and three times with PBS. DY647-C6 detection antibody (2.5 µg/mL, 100 µL) was applied to the wells and the plate was incubated at 37 °C for 1 hour. The plate was then rinsed again three times with PBS Tween20® and three times with PBS. Fluorescence readings were then taken on a Tecan Infinite 200 Spectrophotometer using fixed excitation and emission wavelength of 647 and 677 nm, respectively.

Human IgG NP Assay with Dissolution Amplification.
Anti-hIgG (Fab Specific) polyclonal antibody (10 µg/mL, 50 µL) was added to the wells of a Nunc® MaxiSorp™ 96 well black microtiter plate. The plate was then incubated overnight at 4°C. To prevent non-specific adsorption, 100 µL of 1 % (w/v) BSA and 10 % (v/v) Tween in PBS was added to each well and the plate incubated at 37 °C for 1 hour. To remove any non-adsorbed antibody the plate was then rinsed three times with PBS Tween20® and three times with PBS. Following this, 50 µL aliquots of human IgG from human serum were added in a series of dilutions from 50,000 ng / mL to 0 ng / mL to each well and the plate incubated at 37 °C for 1 hour. The rinse cycle was then repeated to remove any non-specifically bound human IgG. Finally, 50 µL aliquots of anti-hIgG conjugated NPs at 0.5 mg / mL were added to each well and the plate incubated for a further hour at 37 °C in the dark. The plate was again rinsed three times with PBS Tween20® and three times with PBS. Fluorescence readings were then taken on a Tecan Infinite 200 Spectrophotometer using fixed excitation and emission wavelength of 646 and 676 nm, respectively at gains of 100, 130 and 150. In the final step, 150µL of dissolution buffer (0.1M Carbonate Buffer, pH 10.0, 0.5% SDS) was added to each well and the plate incubated for a further hour at 37 °C. Fluorescence readings were again taken using the specifications described previously.

Human IgG Free Dye Immunoassay.
Anti-hIgG (Fab Specific) polyclonal antibody (10 µg/mL, 50 µL) was added to the wells of a Nunc® MaxiSorp™ 96 well black microtiter plate. The plate was then incubated overnight at 4°C. To prevent non-specific adsorption, 100 µL of 1 % (w/v) BSA in PBS was added to each well and the plate incubated at 37 °C for 1 hour. To remove any non-adsorbed antibody the plate was then rinsed three times with PBS Tween20® and three times with PBS. Following this, 50 µL aliquots of human IgG from human serum were added in a series of dilutions from 50,000 ng / mL to 0 ng / mL to each well and the plate incubated at 37 °C for 1 hour. The rinse cycle was then repeated to remove any non-specifically bound human IgG. Finally, 50 µL aliquots of Cyanine5-conjugated mouse anti-hIgG label at 50 µg/mL were added to each well and the plate incubated for a further hour at 37 °C in the dark. The plate was again rinsed three times with PBS Tween20® and three times with PBS. Fluorescence readings were then taken on a Tecan Infinite 200

Spectrophotometer using fixed excitation and emission wavelength of 646 and 676 nm, respectively at gains of 100, 130 and 150.

CRP NP Immunoassay with Dissolution Amplification performed using a point of care style device.

In order to quantify the potential performance in a low cost point of care style device, 0.71 % Cyanine5 (w/w) NPs were tested using a custom built system. This system comprised of a monochrome CCD camera (imaging source DMK 21AU618 Mono), fitted with a BrightLine® fluorescence filter set. A 635 nm 50 mW laser diode excitation source filtered by a BrightLine® single-band bandpass filter with central wave-length of 632 nm was used to excite the fluorophores. The assay was performed in a Nunc® MaxiSorp™ 96 well transparent microtiter plate. The assay protocol was identical to that described in the **CRP NP Immunoassay with Dissolution Amplification** section**.** Fluorescence measurements were performed at each concentration in triplicate both prior to and after dissolution. The fluorescence intensity was measured along a single optical axis, with the sample being illuminated from beneath and fluorescence collected from above the sample well. The fluorescence images were quantified using the open source image processing and analysis software Image-J.

Instrumentation

TEM micrographs were obtained using either JEOL 2100 Transmission Electron Microscope operated at 200 kV or a FEI Tecnai F30 TWIN field emission gun operated at 300kV. Using the JEOL Images were captured digitally using an AMT CCD digital camera. Using the Tecnai F30 images were captured using a Gatan Ultrascan 895 CCD camera, which is a 4-port readout camera with 4Kx4K 15-um pixels. Specimens were prepared by dropping ethanol or aqueous solutions of the NPs onto a formvar carbon coated copper grid. Fluorescence readings were taken on a Tecan Infinite 200 Spectrophotometer using fixed excitation and emission wavelength. For the Cyanine5 and DY647 dyes the excitation wavelengths were set at 646 nm and 647 nm, and the emission wavelengths set at 676 nm and 677 nm, respectively. UV-Vis extinction spectra were measured on a Thermo Scientific™ NanoDrop™ spectrophotometer. NPs were measured in ethanol at 1 mg/mL using a 2 mm path length quartz cuvette. The silica NPs were colloidally stable in ethanol for the duration of the experiment.

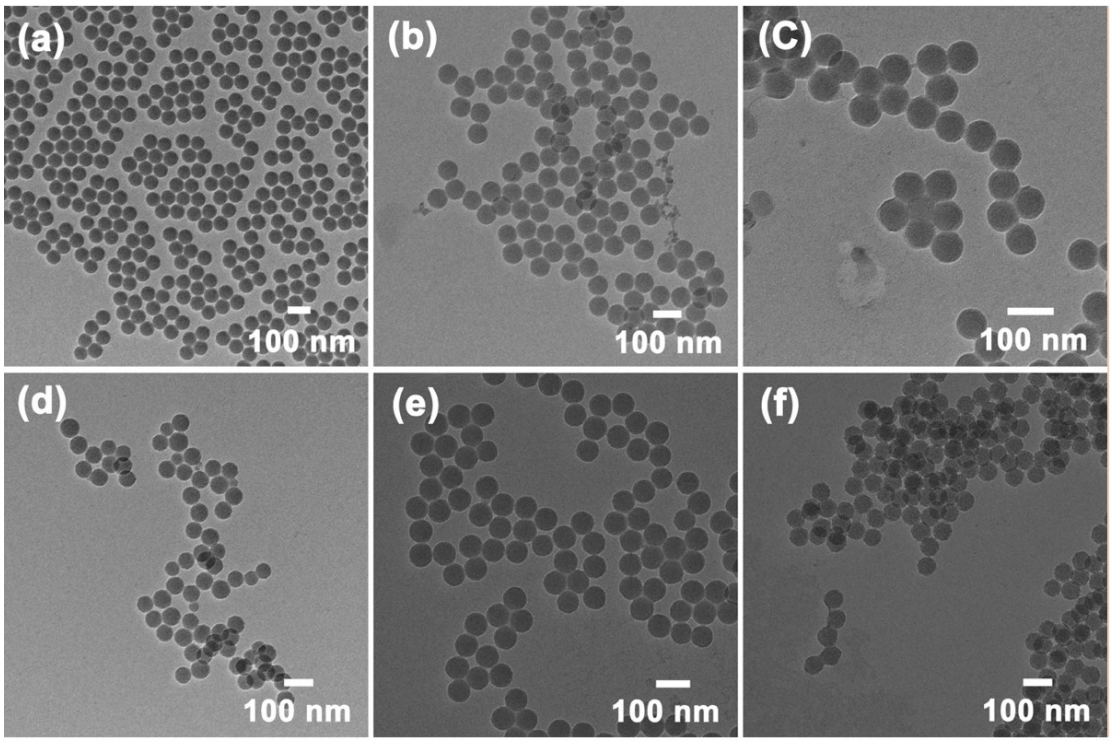

Figure S3) Stability of PEGylated 0.38 % (w/w) Cyanine5 NPs taken at different times (a = 0 mins, b = 10 mins, c = 30 mins, d = 2 hours, e = 24 hours, f = 1 week) whilst stored in phosphate buffered saline.

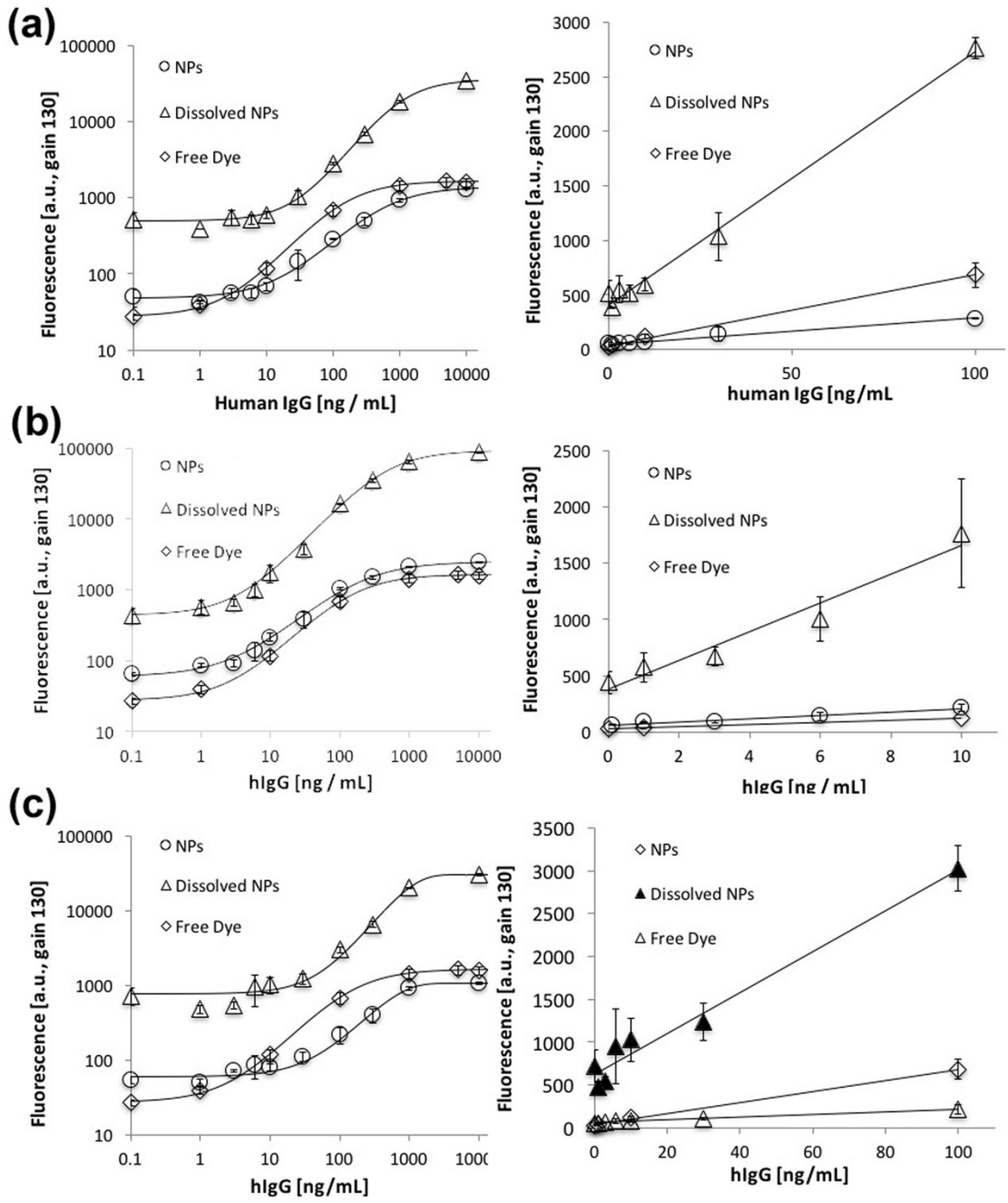

Figure S4) Immunoassay dose response curves for the detection of hIgG using as prepared Cyanine5 dye-doped silica NPs and dissolved NPs compared to results using free dye label displayed over entire dynamic range and at low concentration. The nanoparticles are 24 +/- 5 nm in diameter with a Cyanine5 dye loading of 1.16 % (w/w). The NPs were prepared with three different surface coatings using PEG linker bioconjugation. Sample (a) NPs were coated with hIgG only. Sample (b) NPs were coated with hIgG and BSA. Sample (c) NPs were coated with hIgG and a second PEG group with the aim to reduce non-specific-binding. For all assay NPs were dispersed in colloidal solution containing, 1 % BSA and 0.05 % Tween 20 in PBS.

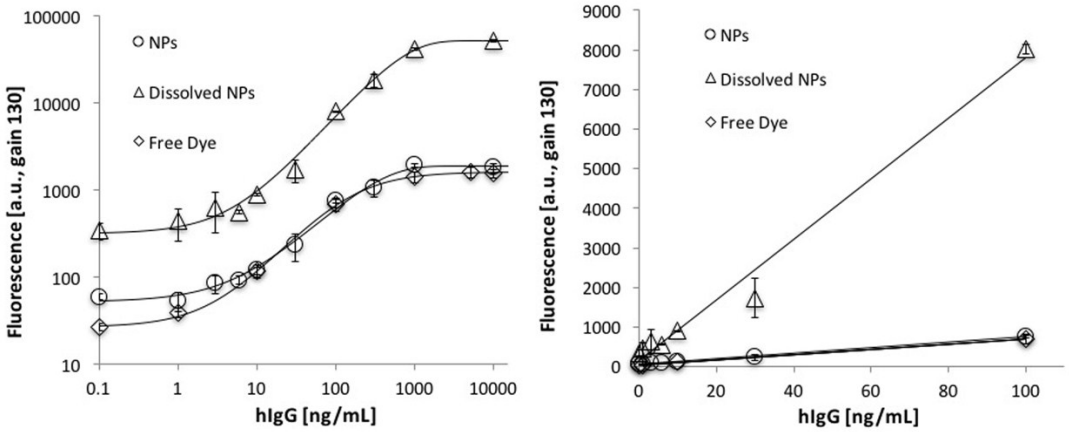

Figure S5) Immunoassay dose response curves for the detection of human IgG using Cyanine5 NPs and dissolved NPs compared to results using free dye label displayed over entire dynamic range (a) and at low concentration (b). The nanoparticles are 18.3 +/- 3 nm in diameter with a Cyanine5 dye loading of 0.99 % (w/w).

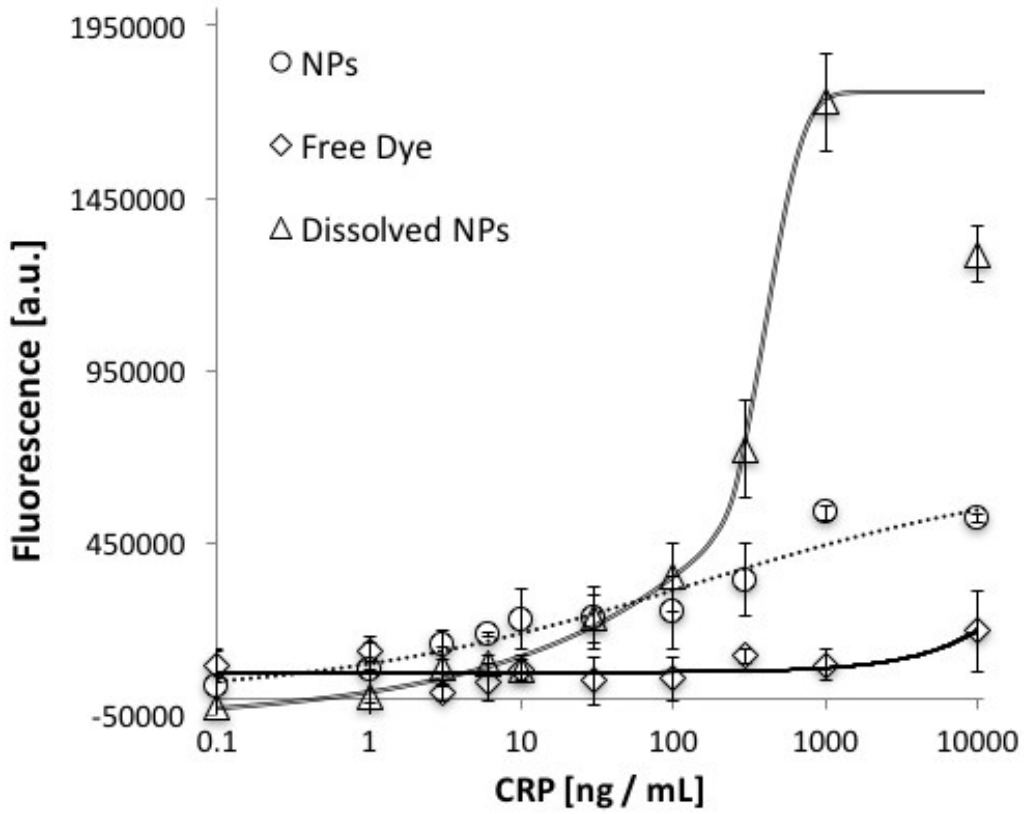

Figure S6) Immunoassay dose response curves for the detection of CRP using NPs and a CCD camera in a POC style instrument set up. Assays using as-prepared NP and dissolved NP labels were compared to results using a free dye label. The nanoparticles are 35.5 +/- 4 nm in diameter with a Cyanine5 dye loading of 0.71 % (w/w).

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