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Supporting Information

‘Overloading’ fluorescent silica nanoparticles with dyes to improve immunoassay performance.

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Contents

1 Materials

Cyclohexane (anhydrous, 99.5%), 1-hexanol (anhydrous, ≥99%), Triton® X-100, aminopropyl trimethoxysilane [APTMS] (97%), tetraethyl orthosilicate [TEOS] (99.99%), ammonium hydroxide solution (28% w/v in water, ≥99.99%), 3-(trihydroxysilyl)propyl methylphosphonate monosodium salt (42% w/v in water) [THPMP], fluorescein isothiocyanate isomer I (≥90%), albumin from bovine serum (lyophilised powder, ≥98%), dextran (from *Leuconostoc spp*, M_r ~40,000), sodium periodate (99.8%), sodium borohydride (≥99%), N,N-dimethylformamide (anhydrous, 99.8%) [DMF], IgG from human serum (reagent grade, ≥95%), 4-morpholineethanesulfonic acid [MES] (≥99%), sodium carbonate (≥99.5%), sodium bicarbonate (≥99.5%), Tween®20, ethanolamine (≥98%), ninhydrin (≥99%), fetal bovine serum were purchased from Sigma Aldrich. Sodium carbonate (0.1M) combined with sodium bicarbonate (0.1M) yielded the pH8.8 (1:9 v/v respectively) and pH10.6 (9:1 v/v respectively) solutions at 37°C used for NP degradation, and are hereafter referred to as 'pH8.8' and 'pH10.6' solutions. Absolute ethanol, phosphate buffer saline tablets (one tablet dissolved in 200mL DI water yields 0.01M phosphate buffer, pH7.4) were purchased from Fisher Scientific. Hereafter, 'PBS' refers to 0.01M PBS, pH7.4. AlexaFluor®647 labeled goat anti-human (H+L) was purchased from Life Technologies. Transparent Nunc Maxisorb 96 well plates were purchased from Fisher Scientific.

2 Dye-loaded silica NP synthesis and functionalisation

2.1 Water-in-oil reverse microemulsion method

Dye-doped silica nanoparticles were synthesized using the quaternary microemulsion method[1]. 2.5mg FITC dye (6.4µmol) and 5.6µL APTMS (32µmol) were reacted together in a dry glass vial in 2mL of 1-hexanol under constant

stirring for 5 hours under light protection. After 5 hours, the FITC-APTMS dye conjugate solution was added to cyclohexane (7.5mL), 1-hexanol (1.133mL), TritonX-100 (1.89g), DI water (480µL) in a 30mL polypropylene bottle under constant stirring. Because the FITC-APTMS solution was based in hexanol, additional hexanol was added such that the total volume of hexanol was consistent through the differently loaded NPs. For example, 3ww NPs used 666µL of dye solution. When 1ww NPs were prepared, 222µL of dye solution was used, followed by the addition of 444µL hexanol. **Table S1** shows the quantities of dye solution and additional hexanol used for each dye-loading concentration. Following the addition of dye solution, 100µL (i.e. 93.3mg) of TEOS was introduced. The 'w/w' values of FITC-APTMS solution added were calculated by comparing to the mass of FITC-APTMS added to the microemulsion to that of 100µL of TEOS. The mixture was allowed to stir for 30mins. 40µL ammonium hydroxide was then added to the reaction mixture to catalyze the nanoparticle synthesis reaction, and the reaction was allowed to proceed for 20 hours under light protection. TEOS (50µL) was added to the reaction mixture. 30mins later, 40µL of THPMP was added and, 5mins later, 10µL APTMS was introduced. To create NPs without amino functionalisation, only 40µL of THPMP was added. The mixture was allowed to stir for another 20 hours. The microemulsion was then broken by adding 30mL of ethanol. The nanoparticles were purified by centrifuging the solution (14,000rpm) for 10mins, removal of the supernatant and subsequent re-dispersion of the NP pellet in fresh ethanol (30mL) via sonication. This centrifuging/sonication step was repeated 3 times. The final NP dispersion was stored at 4°C under light protection.

2.2 Stöber method Preparation of the dye-precursor solution; The FITC-APTMS conjugate was prepared as described elsewhere[2]. Briefly, in a dry vial, fluorescein isothiocyanate (FITC) (39

mg, 0.1 mmol) was mixed with the stoichiometric amount of APTMS (23 µL, 0.13 mmol) in absolute ethanol (100 mL). The mixture was stirred at 40 ˚C for 24 h. The appropriate amount of such prepared FITC-silane precursor solution was immediately used for NP synthesis.

2.2.1 Synthesis of silica NPs 'A FITC Stöber', 'A 2× Stöber', and 'A 5× Stöber' For the preparation of A FITC Stöber', 'A 2× Stöber', and 'A 5× Stöber' FITC-loaded NPs 5 mL, 10 mL or 25 mL (respectively) from original 39 mL of absolute ethanol were replaced by equal amount of the dye precursor solution. Then TEOS (2.8 mL, 12.5 mmol) was added. The reaction was triggered by the addition of NH_4OH (2.8 mL, 25 % (w/w)) water solution was added under vigorous stirring. The reaction was allowed to proceed overnight at RT, after that the nanoparticles were collected by centrifugation at 7800 rpm for 20 min and washed with ethanol (40 mL) (3 ×).

2.2.2 Synthesis of high-loaded NPs 'A 10× Stöber' The precursor solution was prepared in 10 x higher scale – FITC (39 mg, 0.1 mmol) was dissolved in ethanol (39 mL) and APTES (23 µL, 1.3 mmol) was added. The reaction was carried out at 40 ˚C overnight. After that, the mixture was allowed to cool to RT and TEOS (2.8 mL, 12.5 mmol) was added. The reaction was initiated by addition of NH_4OH (2.8 mL, 25 % (w/w)) water solution under vigorous stirring. The reaction was allowed to proceed overnight at RT, after that the nanoparticles were collected by centrifugation at 7800 rpm for 20 min and washed with ethanol (40 mL) (3 ×)

2.3 Releasing dyes for loading quantification The number of dyes encapsulated in the NP loaded at 0.1, 0.25, 0.5, 1, 1.5, 2, 2.5, 3% (w/w) concentrations was quantified. NPs were degraded to release dyes from the NPs such that homo-FRET or aggregation effects could be eliminated. Fluorescein molecules would therefore be free in solution and their fluorescence could be compared to a fluorescence-based calibration curve of known FITC concentrations. 200µg of NPs

were suspended in 1mL of pH10.6 buffer and incubated for 5 hours at 37°C in a Ther-Mix block heater (Vitl Ltd.). After 5 hours, no NP pellet was formed upon centrifugation. TEM images show that severe NP degradation could be seen after 2 hours in pH10.6 solution. It was therefore assumed that the NPs had degraded after 5 hours and fluorescein was free in solution. Using the TEM diameter of the 0.1, 0.25, 0.5, 1, 1.5, 2, 2.5 and 3% (w/w) NPs and density of silica (2.4g cm^{-3}) it was possible to quantify the number NPs per 200µg of NP. Assuming the NPs were spheres, the volume of 1 NP could be calculated using the NP radius calculated from the micrographs in **Figure S2**. Using the density (i.e. mass per unit volume) of silica, the mass of 1 NP was calculated. The number of NPs per 200µg was then calculated. With the number of dyes and NPs known per 200µg of dye-loaded NPs, the quantity of dyes per NP could be found.

2.4 Quantification of amino groups on the NP surface using ninhydrin

In order to find the maximum Ruhemann's Purple absorbance wavelength, a preliminary experiment using ethanolamine was carried out. In an Eppendorf tube, 200µL of 19.1mM ninhydrin was mixed with 200µL of 191mM ethanolamine (both in ethanol) for 15 minutes at 65°C in the block heater (whilst covered with aluminum foil). The solution began to turn purple in colour after 5 mins, but was allowed to turn a stronger purple by being left for a total of 15mins. The sample was then removed from the block heater. 120µL of the solution was taken and an absorbance scan from 450-800nm was performed. The maximum absorbance for Ruhemann's purple was at 580nm. Using the NPs, 1mg of NPs in 200µL ethanol was mixed with 200µL of 19mM ninhydrin in ethanol for 15mins at 65°C in a block heater. The NPs were then centrifuged (14,000rpm, 8 minutes) and the supernatant was collected. 100µL was added to the wells and the absorbance read at 580nm. A calibration curve using APTMS was prepared in order to quantify the average number of amino

groups per NPs. 200µL of APTMS of different concentrations (in ethanol) was mixed with 200µL 19mM ninhydrin (in ethanol) for 15mins at 65°C.

2.5 Creating Ab-NPs using polyaldehyde dextran as a crosslinker We have previously reported the use of polyaldehyde dextran for the attachment of proteins to silica nanoparticles[3]. Briefly, 500µL of 40 mM of sodium periodate (in DI water) was added to 1µmol 40 kDa dextran in a 2 mL centrifuge tube and shaken at 600 rpm for 90mins. The dextran solution was then added to 1mg of NP pellet, ultrasonicated to redisperse the NP in the solution and shaken for 60mins. 500µL of 40 mM of sodium borohydride (in DMF) was added directly into the NP– dextran solution, which was then shaken for 25mins. The sample was then centrifuged (14 000 rpm, 8 min, x2). The NP pellet was dispersed (via ultrasonication) in DI water after the first centrifugation. This was the most effective way of binding dextran to the NPs. After the second centrifugation, the pellet was re-dissolved in 500µL of 40mM of sodium periodate to further oxidize dextran. The dextran-coated NPs were shaken for 30 min (600 rpm), and then centrifuged (14 000 rpm, 8 min, x2). After the second centrifugation, 484µL 0.1M MES pH 4.2 was added to the pellet and sonicated. AlexaFluor647 antibody (16µL, 2mg mL^{-1}) was added and shaken for 4h (600 rpm). 500µL of 40 mM sodium borohydride (in DMF) was added directly into the tube and allowed shake for another 40 min. To purify the antibody-coated NPs, the sample was centrifuged for 8 min at 14 000 rpm, sonicated using an ultrasonic bath and re-dispersed in 1mL PBS (x4). After the final centrifugation, the antibody-coated NPs were re-dispersed in 500µL DI water (2mg mL^{-1}). The particles were then frozen using liquid nitrogen (200µg per aliquot) in a 1% (w/v) solution of BSA (i.e. 100µL of antibody-coated NPs + 100µL 2%(w/v) BSA). Once frozen, the aliquots were then lyophilized overnight in a using a ScanVac Coolsafe freeze drier connected to an Edwards RV12 pump. When dry, the Ab-NP samples were stored at -20°C until further use.

3 NP characterization

3.1 Dynamic Light Scattering

3.1.1 NPs with different dye loading The plain NPs of different FITC loading and Ab-NPs were dispersed at a concentration of 0.1mg mL^{-1} in DI water. Their size (Z-average) and zeta potentials were analysed in a disposable folded capillary cell using Malvern Zetasizer. A minimum of 30 measurements for size and zeta potential was performed for all samples ($N \geq 3$).

3.1.2 Dextran coated- and antibody coated-NPs Dextran coated- and antibody-coated NPs were suspended in water (0.1mg mL^{-1}), and size (Z-average) and zeta potential were measured under similar conditions to those mentioned above.

3.2 Transmission Electron Microscopy

3.2.1 Plain NP size analysis NPs with different dye loading; 3µL of NPs in water (500µg/mL) was added on 'Carbon Films on 400 Mesh Grids Copper' (Agar Scientific) and allowed to evaporate. Images were taken on a Joel JEM-3200FS transmission electron microscope at 100kV magnification. Using ImageJ software, at least 100 NPs per image were analysed statistical values for NP diameter.

3.2.2 NP dissolution Plain NPs (50µg mL^{-1}, 2mL total volume) were incubated in DI water, pH8.8 or pH10.6 solutions at 37°C over time. After particular time points (0h, 2h, 4h), NPs were centrifuged (14,000rpm, 10mins), supernatant was removed and pellet re-suspended in DI water. Two repetitions centrifuging/re-dispersal in DI water were required to remove salts from the solution. 'Clean' NPs were redispersed in 150µL DI water before being added to TEM grids. 3µL

of cleaned NP sample in DI water was added to the TEM grids and allowed to evaporate. DI water was allowed to evaporate and the grids were inserted into the TEM for imaging.

3.3 UV-Vis and fluorescence analysis

A Tecan Infinite M200 Pro Safire microplate reader was used for the majority of absorbance and fluorescence emission measurements. Samples were added to Nunc Maxisorb 96 well plates before being read.

3.3.1 NPs with different dye loading

NPs in DI water (1mg/mL, 100µL per well) were added to the microwell plate and absorbance/fluorescence emission spectra were obtained. A scan range of 400-600nm, and a wavelength step size of 1nm, was employed for the absorbance spectra measurements. For fluorescence emission scans, an excitation wavelength of 436nm was used to scan from 475-600nm with a wavelength step size of 1nm and a gain setting of 55.

3.3.2 Quantification of dye loading

100µL of degraded NP solution (which therefore contained fluorescein released from the NPs) was added to microwells and fluorescence emission intensity was read at $\lambda_{ex}/\lambda_{em}$ = 490/525nm, gain 60). These values were compared to a calibration curve of free FITC in pH10.6 solution.

3.3.3 Ninhydrin

To find the absorbance maximum for Ruhemann's Purple, 100µL of ninhydrin-ethanolamine reaction was added to the wells of a 96 well plate and absorbance was read from 450nm-800nm (1nm step size). For ninhydrin-NP reaction, 100µL of supernatant was added to the wells of a 96 well plate and absorbance read at 580nm (the absorbance maximum of Ruhemann's Purple).

3.3.4 Quantification of antibodies per NP 60µL of purified Ab-$NP_{OPTIMUM}$ and Ab-$NP_{OVERLOADED}$ was added to 96 well plates, and readings for AlexaFluor647 ($\lambda_{ex}/\lambda_{em}$ = 647/677nm, gain 110) and fluorescein ($\lambda_{ex}/\lambda_{em}$ = 490/525nm, gain 55) were used to quantify antibody and NP concentration respectively. These readings were then compared to calibration curves for AlexaFluor647 goat anti-human IgG and FITC-doped nanoparticles. The concentration of antibodies and NPs per well could then be calculate the mass of antibody per mass of NP.

3.3.5 Ab-NP dissolution studies The change in fluorescein fluorescence intensity of Ab-$NP_{OPTIMUM}$ and Ab-$NP_{OVERLOADED}$ was monitored as an indicator of NP degradation. Freeze dried Ab-NPs were removed from the freezer and redispersed in DI water and centrifuged. The supernatant was removed (presumably containing free BSA). The Ab-NP pellets were redispersed in DI water, pH8.8 or pH10.6 solution and added to microwells (10µg/mL or 50µg/mL, 100µL per well). The plate was then added to the microwell plate reader and fluorescein fluorescence intensity read over time. Each well was read at $\lambda_{ex}/\lambda_{em}$ = 490/525nm with a gain setting of 75 at 10min intervals. Room temperature measurement were performed without heating, while 37ºC analysis involved utilizing the plate reader heating capability at 37 ºC for the duration of the measurement. Between measurements the plate was shaken inside the microwell plate reader (orbital shaking frequency of 6mm).

3.3.6 Free FITC emission spectrum A Varian Cary Eclipse Fluorescence Spectrophotometer was used to obtain the emission profiles of free FITC in pH7 DI water, pH8.8 and pH10.6. Using a quartz cuvette, a FITC (2.5µg/mL) emission scan from 470-600nm was performed at λ_{ex}= 455nm using a scan rate of 600nm/min and a gain of 500V.

4 Direct binding assay and subsequent Ab-NP degradation

60μL of human IgG solution in PBS [50nM, 5nM or 0.5nM] was added to the wells of a 96 well plate. 60μL of 1% BSA (w/v) in PBS was added to separate wells as a control as BSA exhibits low non-specific binding. Fluorescent signal generated from these wells solely coated with BSA was considered 'noise'. The plate was incubated at 4°C overnight. The plate was then cleaned by washing each well 5 times (PBS, Tween (0.2% v/v) in PBS, PBS, Tween/PBS, PBS). All wells were then blocked with 120 μL of 1% (w/v) BSA solution and incubated at 37°C for 2 h. The same well cleaning procedure was used as before. 60μL of 200μg/mL of 2 types of freeze dried Ab-NPs [Ab-$NP_{OPTIMUM}$ and Ab-$NP_{OVERLOADED}$] were redispersed in whole FBS, then incubated with the human IgG- or BSA-functionalised wells overnight at 4°C. The wells were then cleaned as mentioned before. Following cleaning, 100μL of either DI water, pH8.8 solution or pH10.6 solution at 37°C was added to the wells with the hypothesis that any Ab-NPs present in the wells would degrade to release fluorescein into solution. Fluorescein was read at $\lambda_{ex}/\lambda_{em}$ = 490/525nm and a gain setting of 75 at approximately 7min intervals. Between measurements the plate was shaken inside the microwell plate reader (orbital shaking frequency of 6mm). Readings for fluorescein were taken overtime with the plate lid on the plate to avoid sample evaporation. Signal-to-Noise ratios were calculated by dividing positive fluorescent signal Ab-NPs with human IgG by the non-specific binding of Ab-NPs with BSA. At least 4 replicates of the direct binding assay (and subsequent Ab-NP degradation) was performed.

5 Supporting Data

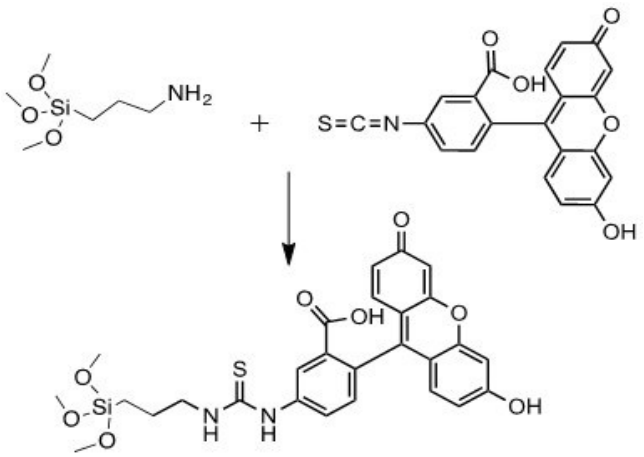

Figure S1. Reacting APTMS with FITC resulted in a FITC-APTMS conjugate linked through a thiourea bond. The methoxysilane groups of FITC-APTMS allowed covalent attachment of the dye conjugate into the silica matrix.

Table S1. Fluorescein-loaded silica NPs were prepared by the water-in-oil reverse microemulsion method. FITC-APTMS conjugate was prepared in hexanol and added to the reverse microemulsion method as a %weight of TEOS. Because 3ww used 666 µL of FITC-APTMS solution (in hexanol), additional hexanol was added to NP batches prepared with 0ww-2.5ww FITC-APTMS such that the total amount of hexanol in each NP batch was the same. Thus the only difference between the NPs batches was the amount of FITC-APTMS.

NP name	% weight vs. TEOS[a)]	Volume of FITC-APTMS added to microemulsion [µL]	Volume of additional 1-hexanol [µL]	Corresponding mass of FITC-APTMS added to microemulsion [mg]	Volume of additional 1-hexanol [µL]
0ww	0	0	666.0	0	666.0
0.1ww	0.1	22.2	643.8	0.09	643.8
0.25ww	0.25	55.5	610.5	0.23	610.5
0.5ww	0.5	111.0	555.0	0.46	555.0
1ww	1	222.0	444.0	0.91	444.0
1.5ww	1.5	333.0	333.0	1.37	333.0
2ww	2	444.0	222.0	1.82	222.0
2.5ww	2.5	555.0	111.0	2.28	111.0
3ww	3	666.0	0	2.74	0

[a)]100 µL of TEOS equals 93.3 mg;

Table S2. Hydrodynamic diameter and zeta potential analysis of the differently dye-doped silica NPs was performed using a Malvern Zetasizer in DI water. All NPs were approximately the same size and, based on their zeta potential values, are colloidally stable. The incorporation of FITC-APTMS into the NP core does not appear to influence the resultant NPs and the reverse microemulsion system must therefore be dependant largely on other factors such as the water-to-surfactant ratio. The polydispersity index (PDI) also indicates that the NPs are monodisperse. TEM micrographs (**Figure S2**) supports the DLS data in demonstrating the NPs are all similar in size.

NP name	**Dye Loading % (w/w)**	**Dynamic Light Scattering**			**TEM**
		Diameter (Z-average) [nm]	PDI	Zeta Potential [mV]	Diameter [nm]
0ww	0	109 ± 11	0.133 ± 0.029	-45 ± 2	47 ± 6
0.1ww	0.1	110 ± 2	0.113 ± 0.034	-46 ± 2	53 ± 5
0.25ww	0.25	113 ± 2	0.083 ± 0.009	-45 ± 3	52 ± 6
0.5ww	0.5	109 ± 7	0.078 ± 0.011	-43 ± 4	51 ± 6
1ww	1	112 ± 8	0.103 ± 0.014	-48 ± 3	46 ± 6
1.5ww	1.5	110 ± 4	0.108 ± 0.026	-46 ± 4	49 ± 6
2ww	2	116 ± 2	0.099 ± 0.015	-47 ± 2	49 ± 6
2.5ww	2.5	114 ± 2	0.085 ± 0.024	-48 ± 1	50 ± 5
3ww	3	113 ± 12	0.091 ± 0.021	-47 ± 2	52 ± 8

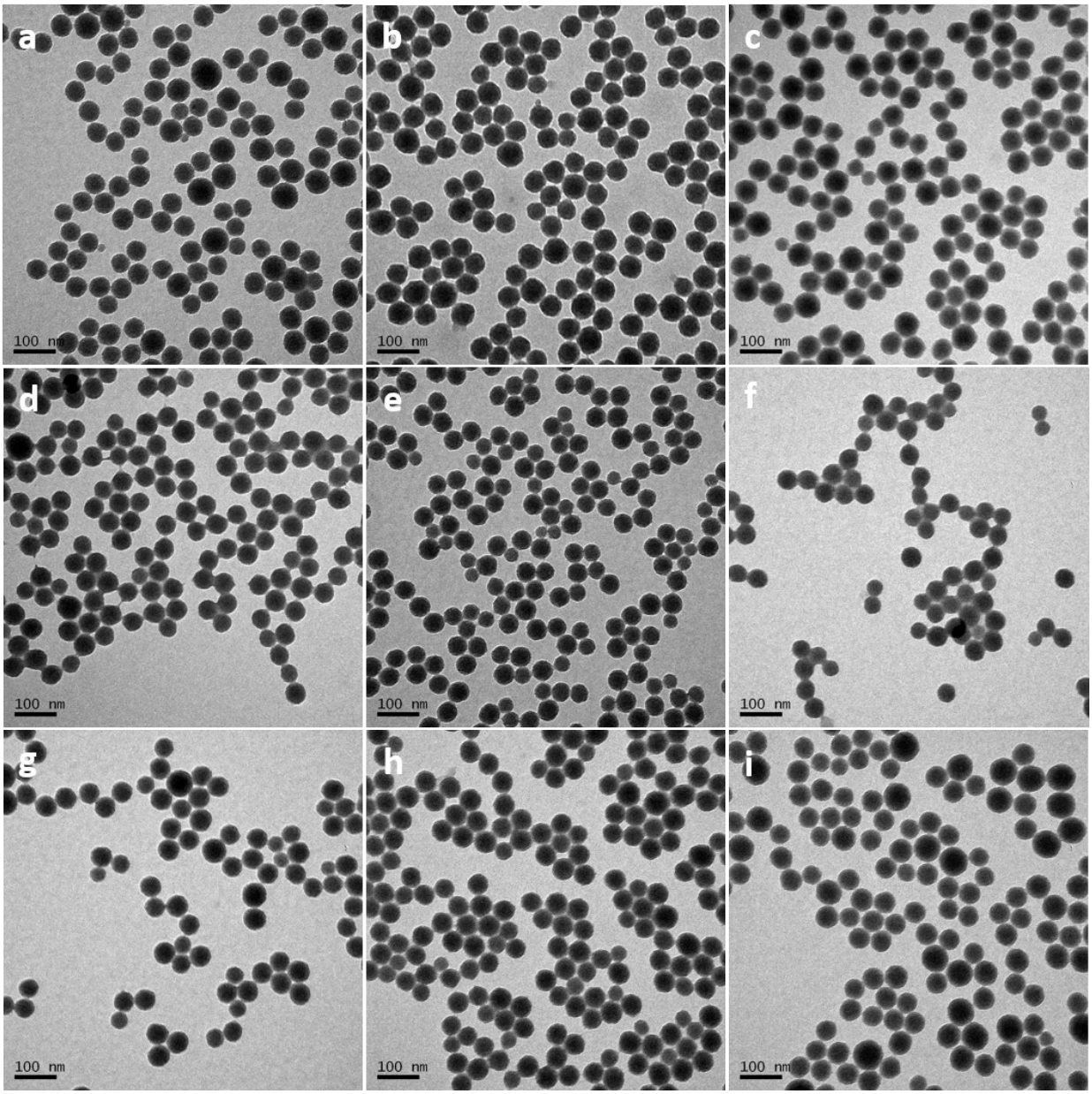

Figure S2. Transmission electron micrographs (100 kV accelerating voltage) of differently dye-loaded NPs. (a) 0ww (b) 0.1ww (c) 0.25ww (d) 0.5ww (e) 1ww (f) 1.5ww (g) 2ww (h) 2.5ww (i) 3ww. All NPs were spherical and there was no apparent change in NP size when different amounts of FITC-APTMS conjugate solution was used in the reverse microemulsion synthesis system. The average diameters for the NPs was (a) 47±6 nm (b) 53±5 nm (c) 52±6 nm (d) 51±6 (e) 46± 6 nm (f) 49±6 nm (g) 49±6 nm (h) 50±5 nm (i) 52±8 nm

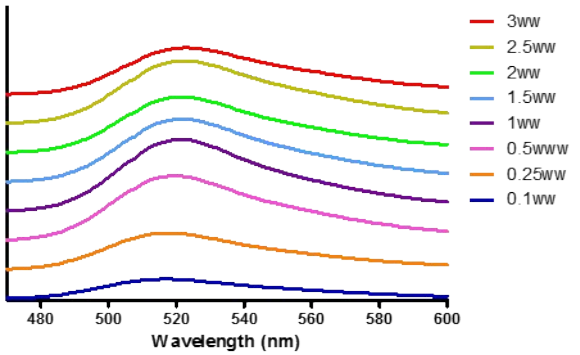

Figure S3. Increasing dye-loading led to a red-shifting of the emission wavelength peak.

Table S3. Ninhydrin reaction was used to quantify the number of $–NH_2$ groups present on the surface of the 1ww NPs ($NP_{OPTIMUM}$) and 3ww NPs ($NP_{OVERLOADED}$). Assuming the NPs were spheres, the number of DDNPs per 1 mg could be calculated as per the Section 2.2 of the main article. The surface area of 1 NP was also calculated using the radius of the micrographs. Using these data it was possible to calculate the number of $–NH_2$ per nm^2 of 1 NP (based on Ruhemann's Purple absorbance). The calculations show that there was little variation in $–NH_2$ coverage between the 1ww and 3ww NPs. The ratio of polyaldehyde dextran crosslinker to $–NH_2$ on the NP surface and shows that there is a large excess of linker per reactive amine.

NP name	Number of NPs per 1mg	'Moles' of NPs per 1mg [pmol]	Surface area of 1 NP [nm^2]	NH_2 per 1mg of NPs [nmol]	NH_2 molecules per nm^2 of NP surface	Dextran:NH_2 during linker attachment
1ww	8.17×10^{12}	13.6	6648	72.9 ± 27.7	0.8 ± 0.3	14 ± 5
3ww	5.67×10^{12}	9.4	8495	47.8 ± 12.0	0.6 ± 0.2	21 ± 5

Table S4. Dynamic light scattering was used to follow the progress of the size (Z-average) and zeta potential of the NPs during dextran and antibody attachment. Fluorescence analysis allow for the mass of Alexa647-labelled antibodies per mass of fluorescein-doped NPs to be calculated.

Dye Loading	NP type	Size (Z-average) [nm]	PDI	Zeta Potential [mV]	Antibodies per NP [µg/mg]
1ww ($NP_{OPTIMUM}$)	NP	112 ± 8	0.103 ± 0.014	-48 ± 3	-
	NP + Linker	119 ± 6	0.187 ± 0.012	-23 ± 3	-
	NP + Linker + Antibody	152 ± 12	0.266 ± 0.058	-33 ± 4	3.39 ± 0.36
3ww ($NP_{OVERLOADED}$)	NP	113 ± 12	0.091 ± 0.021	-47 ± 2	-
	NP + Linker	127 ± 9	0.145 ± 0.039	-22.1 ± 1	-
	NP + Linker + Antibody	166 ± 3	0.214 ± 0.021	-33 ± 3	3.17 ± 0.86

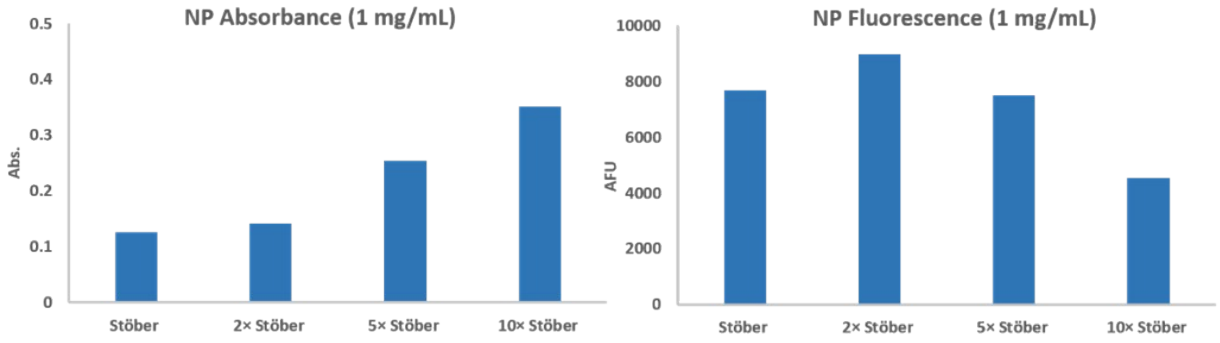

Figure S4. Absorbance and emission spectra of FITC-doped silica NPs produced using the Stöber method. Adding more dye-conjugate during synthesis led to a greater absorbance, and presumably more dyes per NP. However, maximal fluorescence emission was observed for '2×' Stöber NPs. The reduced emission intensity for '5×' and '10×' loading occurred to self-quenching caused by FITC 'overloading'.

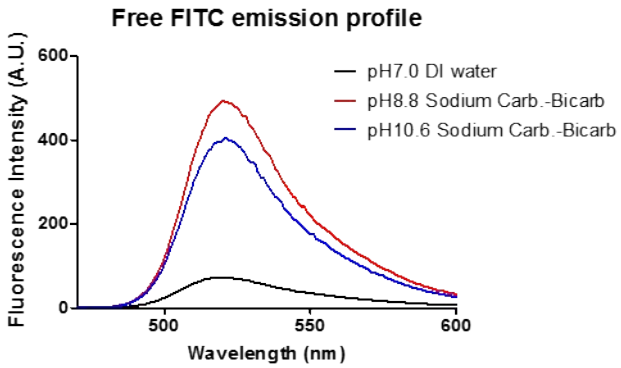

Figure S5. Graph of FITC (2.5µg/mL) emission in water, pH 8.8 and pH 10.6

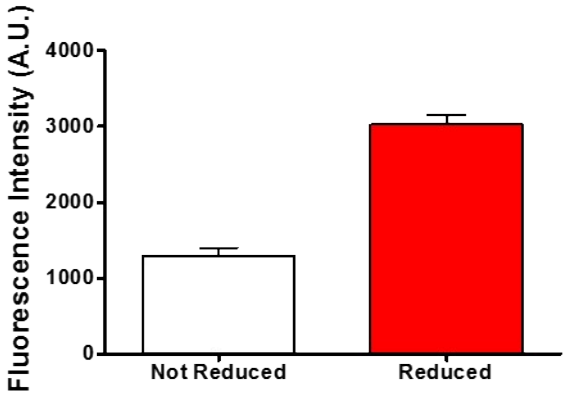

Figure S6. Normalised fluorescence intensity of AlexaFluor647-labelled antibodies bound to the DDNP surface. The use of a reducing step bound 2.4 times more AlexaFluor647-labelled antibodies to surface of DDNPs (red). Without reduction meant the reversible Schiff base used to link dextran and the protein was not stabilized, and less antibody was bound to the DDNP surface (white).

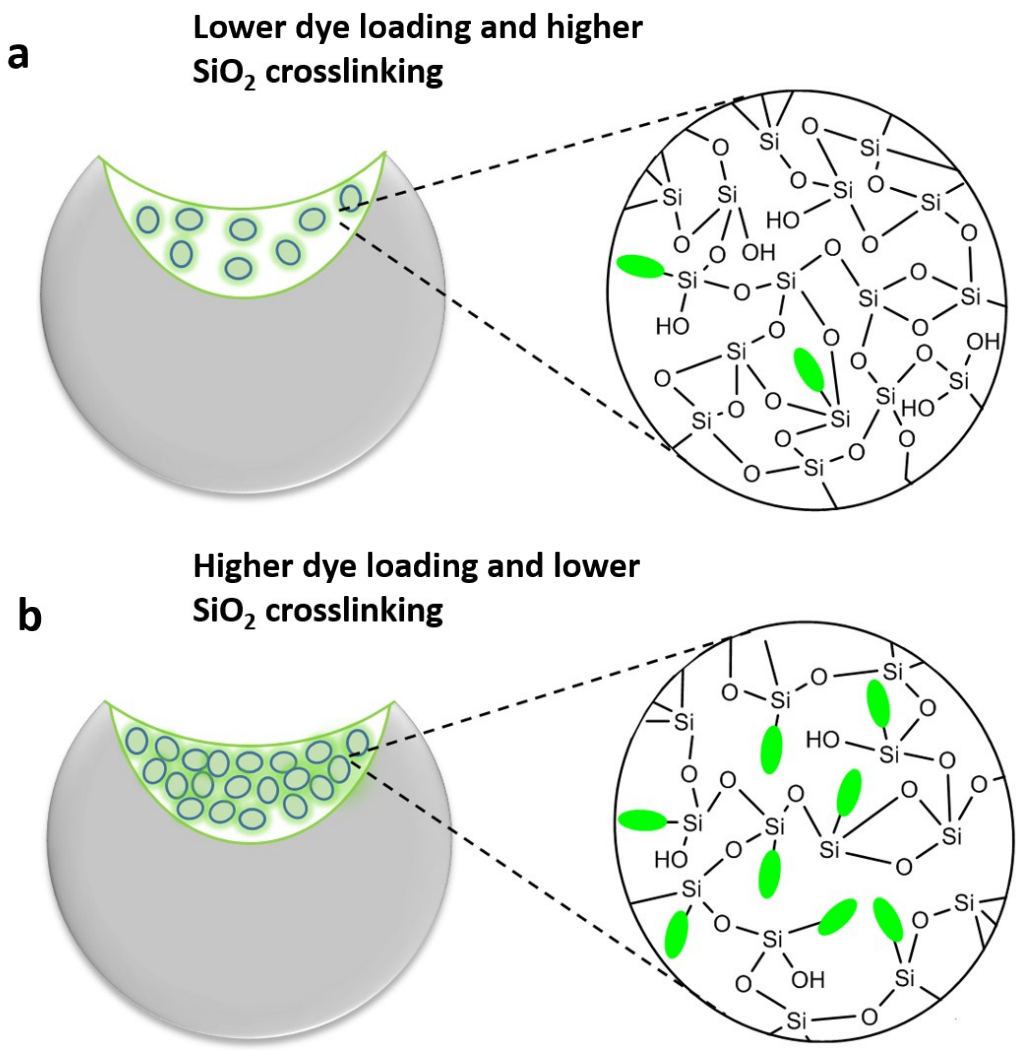

Figure S7. It was hypothesized that higher fluorescein-loading inside the NP core led to a reduction in O-Si-O bonds. **(a)** Lower concentrations of dye-loading led to increased hydrolysis and condensation of TEOS monomers (which have silicon atoms that can form 4 Si-O bonds), and resulted in a high density of O-Si-O bonds in the NP core. **(b)** When more FITC-APTMS was added during NP synthesis, less hydrolysed/condensed TEOS monomers are involved in NP core formation (because FITC-APTMS is also taking part). Because FITC-APTMS can only form 3 Si-O bonds, increasing the dye conjugate results in less O-Si-O units throughout the matrix.

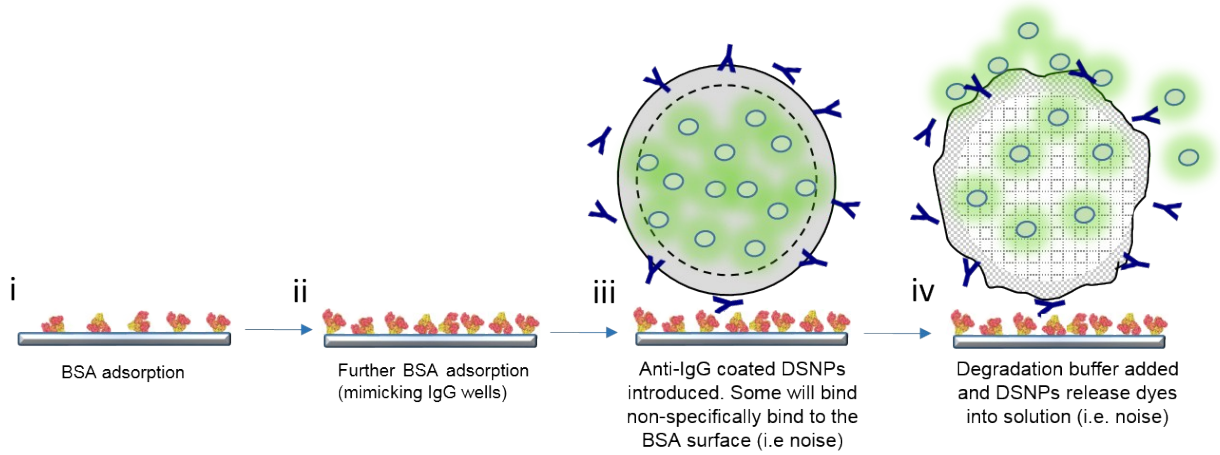

Figure S8. Degradation assay steps using wells solely coated with BSA. The non-specific signal detected in these wells would be considered background 'noise'.

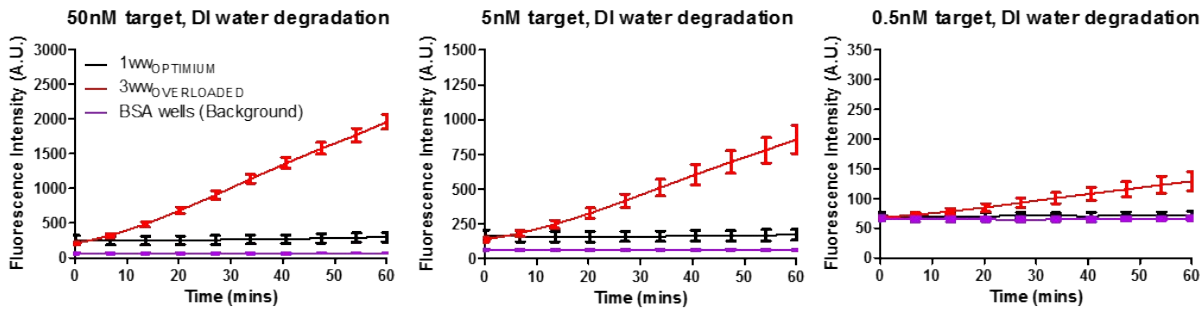

Figure S9. Dissolution assay with DI water as the degradation medium

Table S5. Dissolution assay using pH 7.0 DI water for DSNP degradation. 'Traditional Assay' refers Ab-NP brightness and S/N before dissolution. 'Dissolution Assay' refers to Ab-NP brightness and S/N after dissolution time of 60 minutes.

	Human IgG concentration	50 nM			5 nM			0.5 nM	
		Ab-NP_{OPTMUM}	Ab-$NP_{OVERLOADED}$		Ab-NP_{OPTMUM}	Ab-$NP_{OVERLOADED}$		Ab-NP_{OPTMUM}	Ab-$NP_{OVERLOADED}$
Brightness	Traditional Assay	253.33	204.63		166.17	136.63		70.67	68.38
	Dissolution Assay	300.33	1961.80		171.50	858.50		72.17	129.00
	Signal Enhancement Factor	1.19	9.59		1.03	6.28		1.02	1.89
S/N	Traditional Assay	3.75	3.03		2.46	2.02		1.05	1.01
	Dissolution Assay	4.43	28.93		2.53	12.67		1.06	1.90
	S/N Enhancement Factor	1.18	9.55		1.15	6.27		1.01	1.88
	$Traditional_{OPTIMUM}$ v $Dissolution_{OVERLOADED}$	1 : 7.71			1 : 5.15			1 : 1.81	

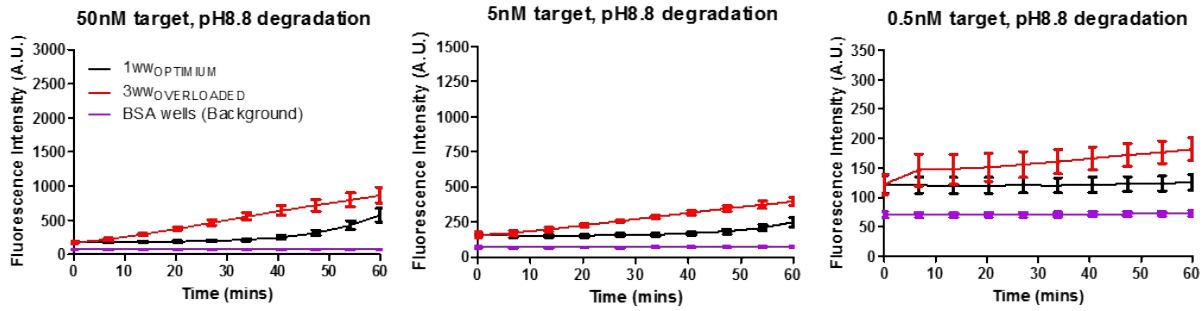

Figure S10. Dissolution assay with pH 8.8 as the degradation medium

Table S6. Dissolution assay using pH8.8 sodium carbonate-bicarbonate for DSNP degradation. 'Traditional Assay' refers Ab-NP brightness and S/N before dissolution. 'Dissolution Assay' refers to Ab-NP brightness and S/N after dissolution time of 60 minutes.

	Human IgG concentration	50 nM			5 nM			0.5 nM	
		Ab-NP_{OPTMUM}	Ab-$NP_{OVERLOADED}$		Ab-NP_{OPTMUM}	Ab-$NP_{OVERLOADED}$		Ab-NP_{OPTMUM}	Ab-$NP_{OVERLOADED}$
Brightness	Traditional Assay	185.11	170.33		157.78	158.67		121.89	122.22
	Dissolution Assay	576.88	876.00		250.56	397.56		125.78	181.50
	Signal Enhancement Factor	3.12	5.14		1.59	2.51		1.03	1.49
S/N	Traditional Assay	2.62	2.41		2.23	2.25		1.73	1.73
	Dissolution Assay	7.92	11.91		3.44	5.46		1.73	2.48
	S/N Enhancement Factor	3.02	4.94		1.54	2.43		1.00	1.43
	$Traditional_{OPTIMUM}$ v $Dissolution_{OVERLOADED}$	1 : 4.55			1 : 2.45			1 : 1.43	

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