Electronic Supplementary Information

Controlling colloidal stability of silica nanoparticles during bioconjugation reactions with proteins and improving their longer-term stability, handling and storage

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Bioconjugation protocol using PAMAM Dendrimer G4.5

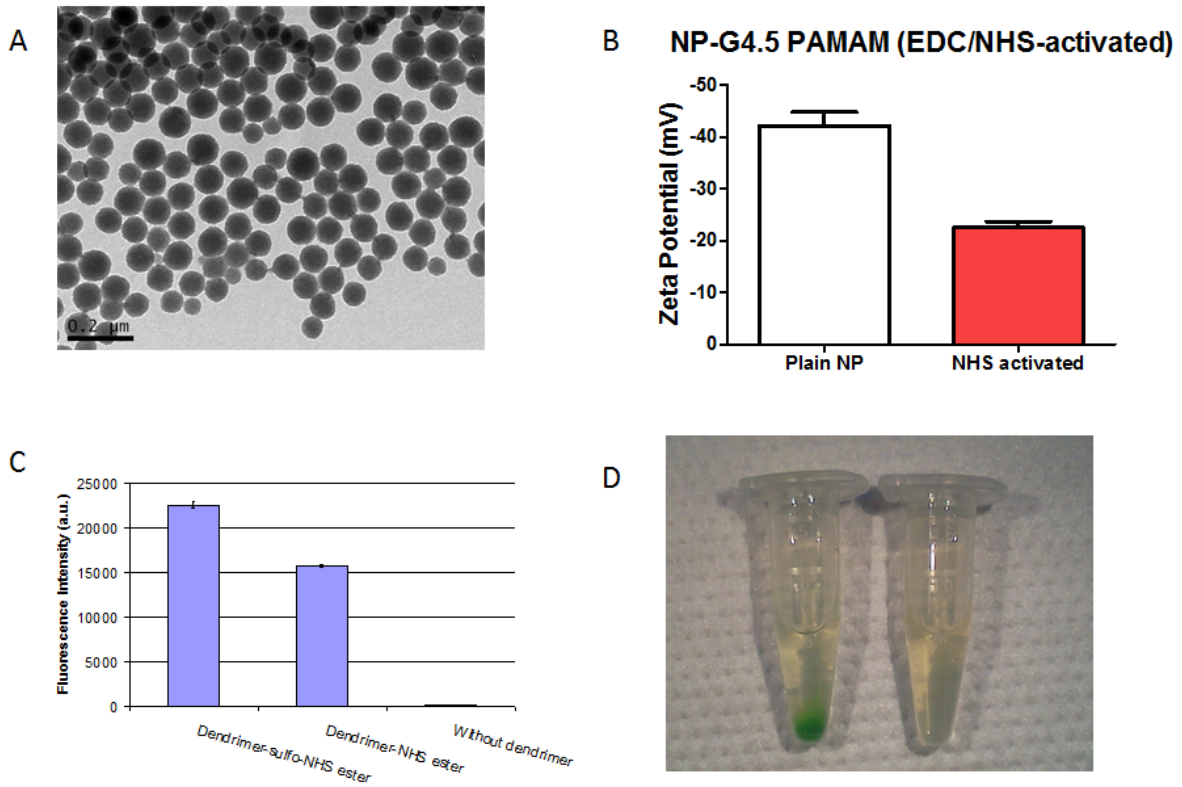


Figure S.1 (A) TEM picture of the silica nanoaprticles. Using ImageJ software, the morphology of the nanoparticles was spherical and their size was calculated to be 80nm ± 12nm. (B) Zeta potential measurements performed in DI water of plain nanoparticle (left) and EDC/NHS activated PAMAM-coated NPs (C) Fluorescence intensities of labelled BSA immobilized on the NP surface using activated PAMAM dendrimer; (D) Photograph of NP samples prepared by NHS/EDC chemistry (left) and sulfo-NHS/EDC chemistry (right) after stored overnight at 4˚C.

Conjugation of anti-CRP

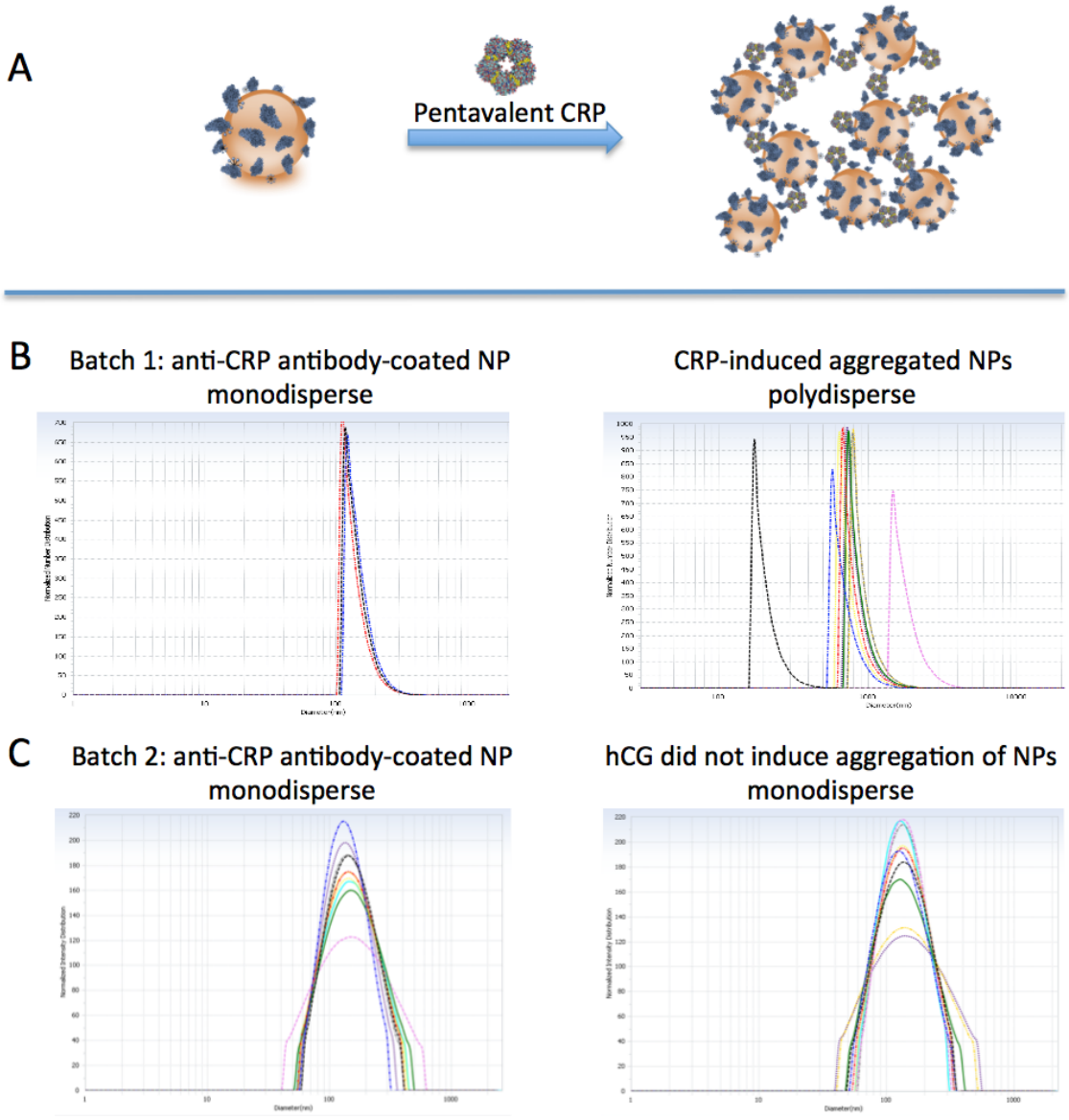


Figure S.2 (A) Schematic illustration of the experiment – the aggregation of Anti-CRP coated NPs was induced by the addition of pentavalent CRP; (B) (Left) Anti-CRP-coated NPs were monodisperse in DI water over 3 hours in the absence of CRP (Right) Evolution of the NPs size measured by DLS illustrating the agglomeration of the sample. NPs started to aggregate as soon as CRP was added into the sample (black line – original size before CRP addition); (C) Separate batch of anti-CRP-NPs measured over the course of 3 hours in DI water (left) and upon addition of non-specific protein, human chorionic gonadotropin (hCG), showing negligible effect of particle aggregation (right)

Initial Dextran Binding Studies

The procedure for dextran binding follows a similar procedure to that of the established dendrimer conjugation method; namely there is a linker activation step and a mixing with nanoparticles step followed by reacting with antibodies

		Zeta Potential (mV)		
Sodium Periodate	**Sodium Borohydride**	**4mM**	**40mM**	**400mM**
DI Water	DI Water	-41.8 ± 1.3	-38.0 ± 1.1	-37.6 ± 2.5
DI Water	DMF	-29.4 ± 2.0	-19.3 ± 1.9	Unable to Sonicate
DMF	DI Water	-34.1 ± 5.0	-23.6 ± 0.9	Unable to Sonicate
DMF	DMF	-50.0 ± 13.1	-44.4 ± 4.6	-32.0 ± 4.2

This table summarises the initial experiments done to investigate the best conditions to successfully bind dextran to the nanoparticles. Dextran is a neutrally charged molecule and therefore it's binding to the nanoparticle results in a lowering of the particle's overall zeta potential.The procedure that resulted in the largest drop in zeta potential indicated the largest amount of dextran bound to the nanoparticle surface. It was assumed that the largest the amount of dextran bound to the nanoparticle surface held the most potential for binding the highest amount of antibodies.

1µmol (40mg) of 40kDa dextran was oxidised using different concentrations (4mM, 40mM or 400mM) of sodium periodate in DI water or DMF for 1hr 45mins.The addition of 500µL of 4mM, 40mM or 400mM sodium periodate to the dextran meant the addition of 2µmol, 20µmol or 200µmol of oxidising agent respectively. The oxidised dextran was then added to 1mg of nanoparticle pellet, sonicated and allowed to react for 1hr 50mins. Sodium borohydride was then added directly into the reaction mixture (in an equivalent concentration to that used for the reduction). For example, if 500µL of 4mM sodium periodate was used for oxidation, then 500µL of 4mM sodium borohydride was used for reduction. The reduction step was allowed to proceed for 45mins. It was found that by oxidising dextran with 40mM DI water, mixing with NPs and then reducing with 40mM sodium borohydride in DMF resulted in the biggest reduction in nanoparticle zeta potential and therefore the largest number of dextran molecules on the surface of the nanoparticle

Decreasing Timescale to Bind Dextran

The total time for dextran conjugation was 4hr 20mins (260mins) and efforts were made to reduce this time for practical reasons, and also because dye molecules are known to leech from the silica matrix in aqueous solutions, which leads to an overall reduction in nanoparticle brightness. The oxidation time and the reaction time with the nanoparticles were adjusted, while the reduction time was set to 25minutes. Oxidation times of 30mins, 1hr and 1.5hrs were trialled and 30min and 1hr were used for dextran-NP reaction. The dextran oxidation and dextran-NP reaction times influence the degree to which dextran is bound to the NP surface, and is seen as a drop in NP zeta potential. The conditions best suited to achieving this large decrease, which is attributed to larger amounts of dextran bound to the NP surface, is oxidation in 40mM sodium periodate (in DI water) for 1.5hrs, followed by dextran-NP mixing for 1hr and reduction for 25mins using 40mM sodium periodate (in DMF). This improved dextran-NP conjugation procedure took 2hrs 55mins (175mins).

Below is the zeta potential analysis from these experiments

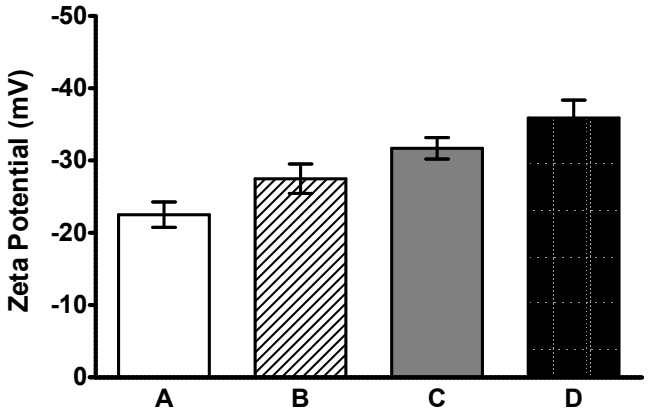


Figure S.3 A -22.5mV [Oxidation for 1.5hrs, mixed with NPs for 1hr, reduced for 25mins. **B** -27.5mV [Oxidation for 1hrs, mixed with NPs for 1hr, reduced for 25mins] **C** -31.7mV [Oxidation for 30mins, mixed with NPs for 1hr, reduced for 25mins] **D** -35.9mV [Oxidation for 1.5hrs, mixed with NPs for 30mins, reduced for 25mins]

The effect of the surface chemistries on the NP charge and size

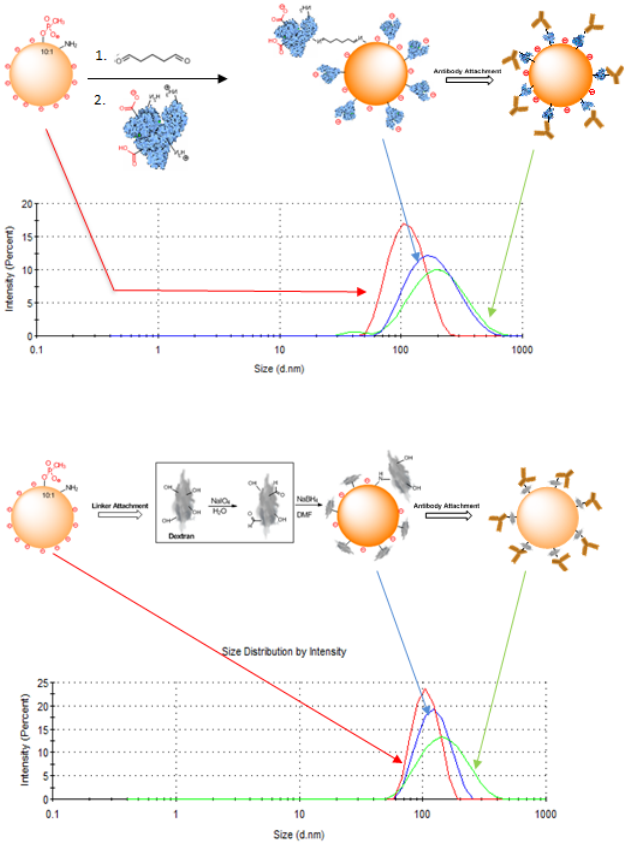


Figure S.4 Evolution of the NP size in the various stages of the bioconjugation reaction using BSA (top) and dextran (bottom).

Antibody Binding

Considering that there were 4 dye molecules per antibody (Alexa Fluor 647 goat anti-human IgG) and, because a whole IgG molecule contains approximately 83 lysine residues (Y. H. Tan, M. Liu, B. Nolting, J. G. Go, J. Gervay-Hague and G.-y. Liu, *ACS Nano*, 2008, 2, 2374-2384), it is unlikely that potential lysine binding sites occupied by dye molecules can strongly influence the binding efficiency of the proteins to the NPs.

Fluorescence Readings

Using Bangs Lab Technical Note 205, the amount of proteins needed to achieve high nanoparticle surface coverage was determined from the formula:

$$S = \left(\frac{6}{\rho_s d}\right) C$$

where S = amount of protein required to achieve surface saturation (mg protein/ g nanoparticle), ρ_s is solid sphere density (which in this case in 2.4g/cm^3 for silica), d is the mean particle diameter (which, from TEM, was calculated to be 80nm) and C is the capacity of sphere surface for a given protein (mg protein/ m^2 of sphere surface), which is approximately 2.5mg/m^2 for IgG molecules and 3mg/m^2 for BSA.

Linker	**Antibodies per NP**	**Active Surface Area [NP surface covered by antibody]**
PAMAM (sulfo-NHS)	120 ± 9	60 ± 5
Dextran	47 ± 14	24 ± 7 %
BSA	78 ± 6	39 ± 3%
Adsorbed Antibody	74 ± 10	37 ± 5%

$4\pi r^2$ = surface area of sphere = $4\pi(40 \times 10^{-9}m)^2 = 4\pi (1.6 \times 10^{-15}m^2) = 2 \times 10^{-14}m^2$, Area of IgG = $100nm^2 = (100)(10^{-18}) = 1 \times 10^{-16}m^2$

For PAMAM:
Area of 120 Antibodies = $(120)(1 \times 10^{-16})\ m^2 = 12 \times 10^{-15}m^2$
% NP surface area coated with antibody = $[(12 \times 10^{-15}m^2)/ (2 \times 10^{-14}m^2)] \times 100 = 60\%$
120 ± 12 Antibodies = 60 ± 6 % of NP surface coverage

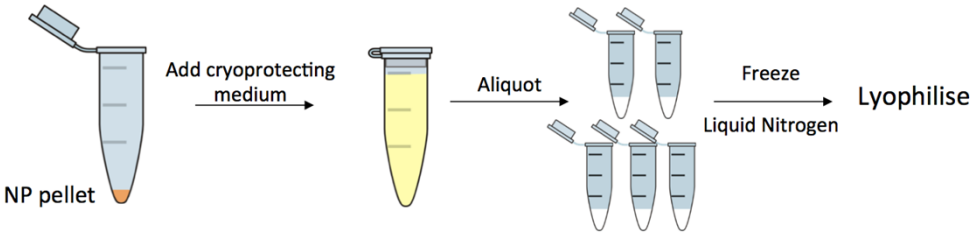
For dextran:
Area of 47 Antibodies = $(47)(1 \times 10^{-16})\ m^2 = 4.7 \times 10^{-15}m^2$
% NP surface area coated with antibody = $[(4.7 \times 10^{-15}m^2)/ (2 \times 10^{-14}m^2)] \times 100 = 24\%$
47 ± 14 Antibodies = 24 ± 7 % of NP surface coverage

For BSA:
78 Antibodies = $(78)(1 \times 10^{-16})\ m^2 = 7.8 \times 10^{-15}m^2$
% NP surface area coated with antibody = $[(7.8 \times 10^{-15}m^2)/ (2 \times 10^{-14}m^2)] \times 100 = 39\%$
78 ± 6 Antibodies = 39 ± 3% of NP surface coverage

For Adsorbed Antibody:
74 Antibodies = $(74)(1 \times 10^{-16})\ m^2 = 7.4 \times 10^{-15}m^2$
% NP surface area coated with antibody = $[(7.8 \times 10^{-15}m^2)/ (2 \times 10^{-14}m^2)] \times 100 = 37\%$
74 ± 10 Antibodies per NP = 37 ± 5% of NP surface coverage

Long term storage and manipulation of Ab-NPs

1. Disperse NPs (plain and Ab-NPs) in a 'cryoprotectant' media (such as sugars, BSA, small zwitterionic molecules etc.), aliquot, freeze and freeze-dry



2. Re-disperse dried sample in biologically relevant media (phosphate buffer saline, cell culture media)
3. **Successful re-constitution of the of the shipped samples should be achieved by simple manipulation, e.g. shaking with hand and the NP samples should be stable for minimum of 2 hours.**

Figure S.5 Three steps describing the proposed protocol for NPs handling. The specific condition is that the end-user should be able to re-dissolve the Ab-NPs in their own lab environment without excessive manipulation with the sample (e.g. ultrasonication, vortexing etc.). In an ideal scenario, a solid formulation of the Ab-NP sample should be reconstituted in the desired solvent by simply shaking it in hand.

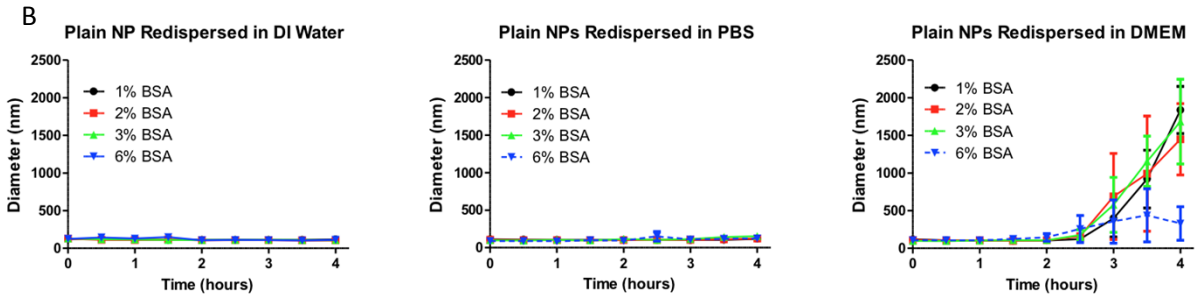


Figure S.6 (A) (left) Photograph of solid formulations of the nanoparticles after being freeze-dried from a 20% (w/v) sucrose solution containing increasing amounts of BSA (0, 1, 2, 3, 6% w/v). Sucrose had a destabilizing effect on the NPs, which was visibly evident from the NP pellet. Increasing amounts of BSA led to a more stable solid-form sample. NPs with sucrose appeared 'melted.' It was impossible to redisperse the NPs back to their original size without ultra-sonication. Increasing concentration (Right) When freeze-dried in BSA solutions (1, 2, 3, 6% w/v) resulted in visually stable solid-form samples. (B) The evolution of the size of the 'plain' NPs that were freeze-dried from a solution containing BSA (1-6% w/v), measured in different aqueous media. Even in

the 'enriched' DMEM culture medium, the NPs retained excellent colloidal stability in 1% w/v BSA solution for 2.5 hours.

The function of Ab-NPs probed by immunoassay

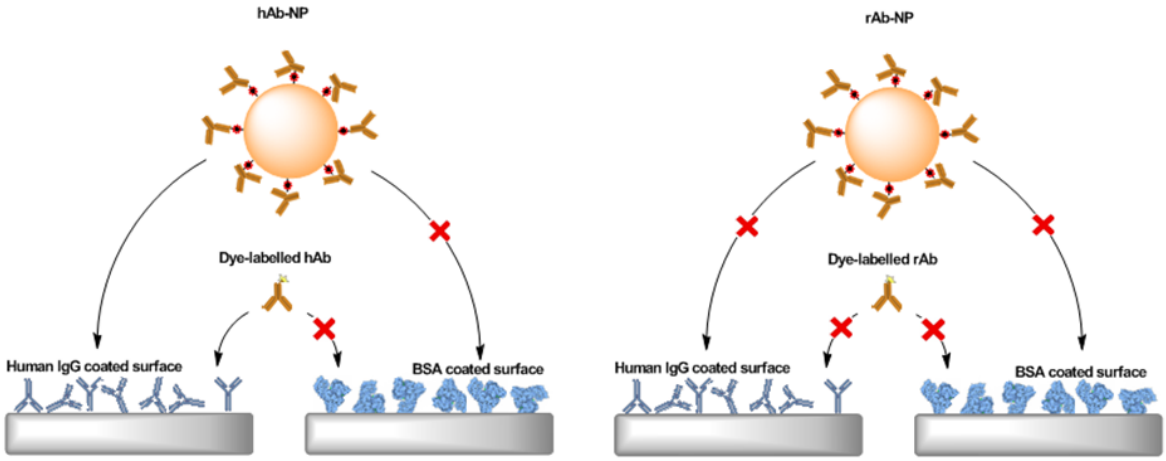


Figure S.7 Cartoons illustrating the design of the immunoassay experiments. 96-well plate format was used. Prior the experiment, wells were coated with either human IgG or BSA. The specific binding was measured as the signal in wells containing human IgG coated surface and incubated with goat anti-human antibodies (either free labelled-antibodies or antibody-coated NPs). The non-specific binding was determined as the signal in wells containing human IgG coated surface and incubated with goat anti-rabbit antibodies (either free labelled-antibodies or antibody-coated NPs). As a control, wells the surface of some of the wells was also coated with BSA. No binding was expected in those wells. All measurements were performed in 4 replicates.