Chemical Quenching- and Physical Blocking-Based Method To Minimize Process-Mediated Aggregation of Antibody-Crosslinked Nanoparticles for Imaging Application

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EXPERIMENTAL

SiNP synthesis and functionalization:

NIR664 dye-doped silica nanoparticles (NPs) were synthesized, characterized and optimized as previously reported by us [1, 2].

Prior to functionalization the NPs were centrifuged and dried under a stream of nitrogen to evaporate any traces of solvent (ethanol). Afterwards, NPs were weighed and a concentration of 20 mg/mL was prepared in the desired solvent as will be described. The optimization of silane-mediated functionalization of NIR664 dye-doped NPs has already been reported by our group with amino-silane as a model silanization agent [2].

Functionalization with amino-silane:

NPs, at the desired concentration, were incubated in a solution of 3-aminopropyltriethoxy silane (APTES) (2% (v/v) in 90% (v/v) ethanol with deionized water) for 3 hours. The functionalized NPs were then washed five times with ethanol. Functionalized NPs obtained after the last wash were stored in ethanol for further analysis.

Functionalization with carboxy-silane:

NP stock was dissolved in deionized water because of the insolubility of carboxysilane in ethanol. Similar to amine functionalization, the dispersed NPs were incubated with carboxyl silane (2% (v/v)) for a period of three hours. These NPs were also processed as above and were stored in ethanol.

Antibody cross-linking

Carboxyl groups on NPs were first activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDC) as reported previously, in 100mM MES buffer at pH 4.7 [2]. Following EDC-activation, the antibodies (15 mg/mL)/ antibodies (anti-HRP antibody for HRP-coated surface and anti-CD41 antibody for the platelet-coated surface at a concentration of 5 µg/mL)

were incubated with the NP solution and continuously stirred for 3h. The conjugates were then washed with PBS for five repeated centrifugations as above.

Sequential centrifugation:

The chemically quenched and BSA-blocked antibody-conjugated NPs were subjected to sequential centrifugation. The NP samples were centrifuged at 67 X g for 2 min in the first round. The supernatant from this round was then centrifuged at 268 X g for 2 min. The same process was performed at 419 and 603 X g. The pellets collected at each step were re-dispersed in optimized PBS solution of specific strength and subjected to mild sonication.

Zeta potential measurement:

Zeta potential for the AfNP and CfNP samples was measured by a Beckman Coulter Instrument by using a flow cell. The averages reported in the Results section were obtained from three measurement sets as analysed by the DelsaNano software where each measurement was the average of 50 individual measurements performed at various positions (10 measurements each in 5 positions) in the flow cell that is set as default.

Fluorescence microscopy:

HRP-based assay: HRP was coated on thin cover-slip glass slides at a concentration of 5 µg/mL at 37 ^{o}C for 2 h. These slides were then rinsed thoroughly with 0.1M PBS, pH 7.2 followed with blocking using 1% (v/v) BSA in PBS for 1 h. These slides were then rinsed thoroughly with PBS. These plates were then incubated with anti-HRP antibody-conjugated Si NPs, which was subjected to chemical quenching, BSA-blocking and gradual centrifugation. Following the HRP experiments platelets were probed with the optimum conditions obtained from HRP experiments with anti-CD41 antibody-conjugated SiNPs. A fibrinogen-coated grid-based platform was developed as described by Basabe-Desmonts et al. [3] and employed for

platelet capture assay. Platelets, by virtue of their biological activity interact with fibrinogen thus got immobilized on surface. Afterwards, the captured platelets were washed with 1mM PBS, pH 7.2 supplemented with 10mM glucose. Nanoparticle-antibody conjugates prepared by the developed recipe were then subjected to probe CD41 marker on platelet surface.

Image-J-based analysis

A 3D profile of the fluorescence intensity was created using interactive 3D surface-plot plug-in. Amount of the intensity of the fluorescence is an indirect measure of number of NPs. It is so because fluorescence is a characteristic of the dye but the dye is inside the NPs therefore, each NP contributes towards the overall fluorescence. In the 3D plots, aggregates were characterized on the basis of the thickness of the peaks on XY-plane and their height on Z-plane, which corresponds to the overall brightness of the cluster [4]. An in-depth analysis of cluster sizes can be performed by a combination of various functions of interactive 3D plug-in, such as smoothing along with Z-scale maximum and minimum [5]. In all the analyses of HRP-related probing experiments, a grid-size of 256 was kept constant along with a smoothing of 0.0; whereas, Z-scale was arbitrarily kept at 0.46 with a Max at 15 and Min at 12% until otherwise stated. The rationale for choosing these Z-scale parameters was to resolve individual peaks in order to make an appropriate comparison. For platelet-based analysis, the Z-scale was kept at 0.46 with a Max of 36 and Min of 0%.

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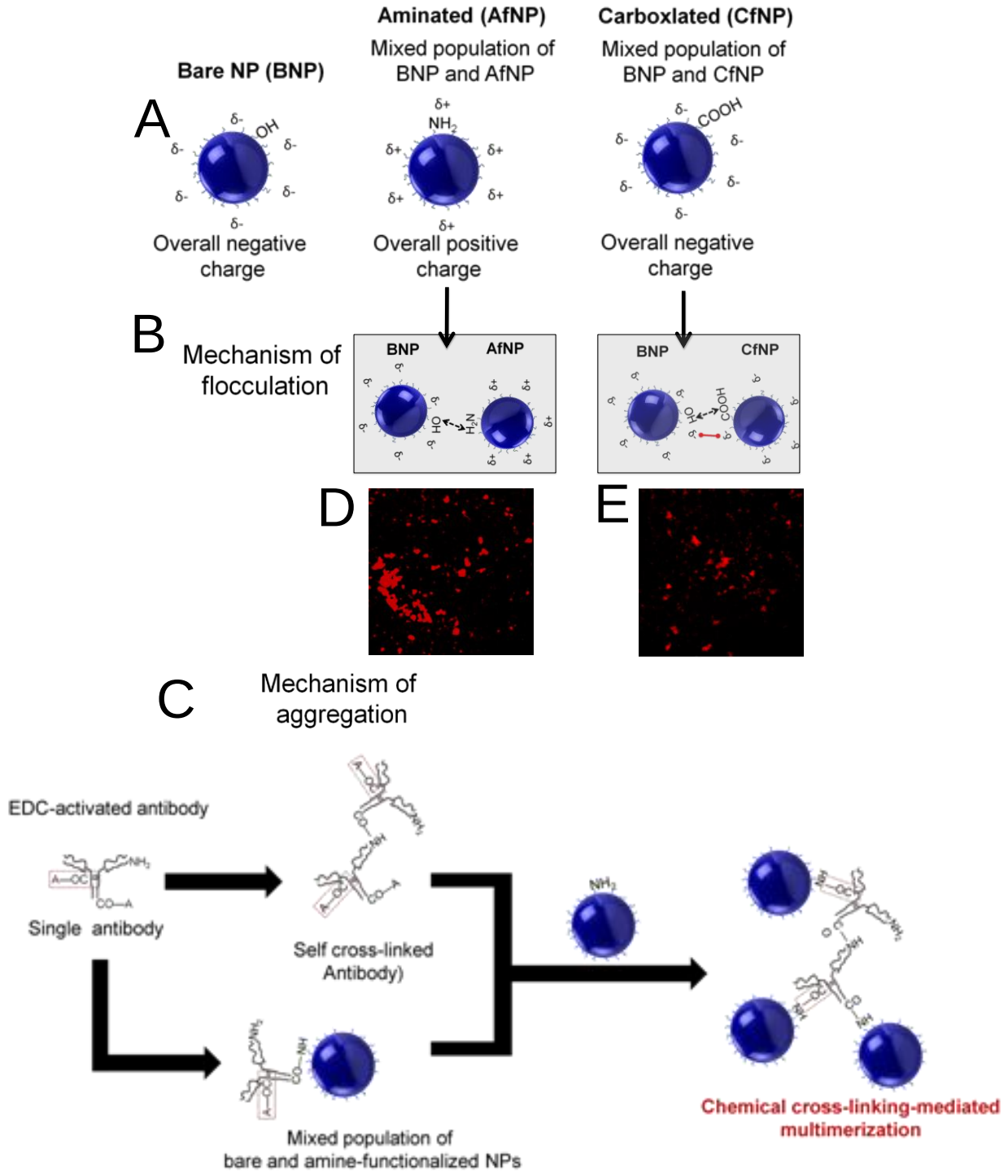
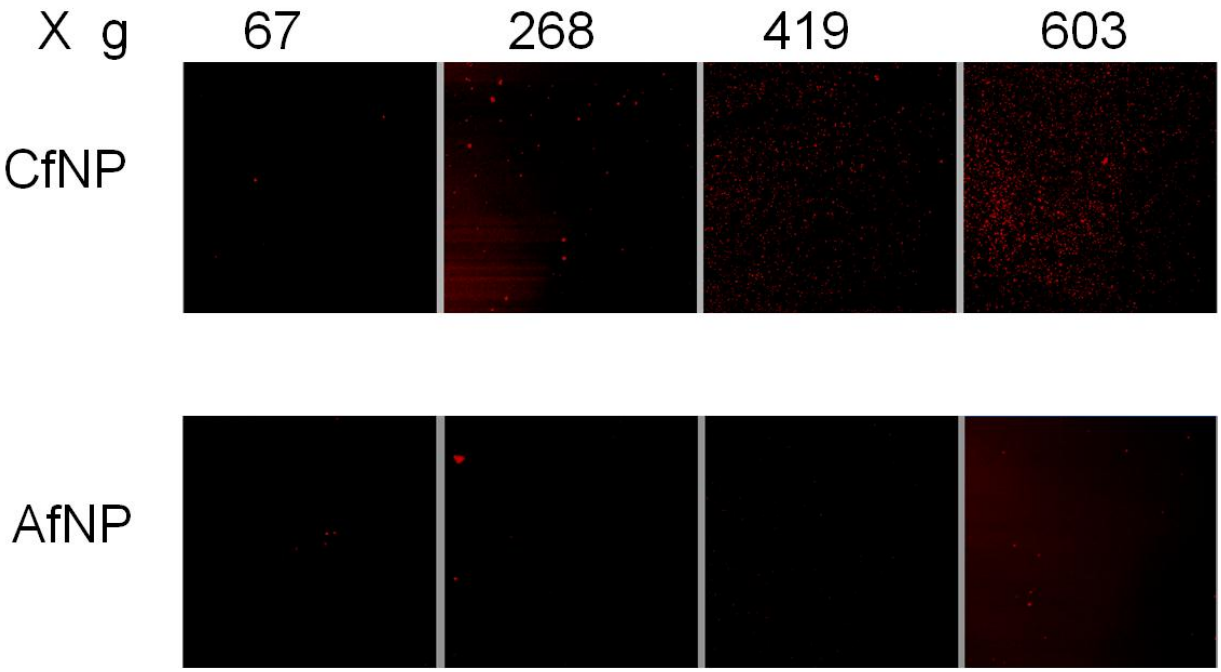
Supplementary Table 1. Zeta potential of NPs in various solvents and at different pHs.

Solvent	NP sample	Zeta potential (mV)
DIW		
	Bare	-38
	Amine-functionalized	29
	Carboxy-functionalized	-29
Ethanol		
	Bare	-36
	Amine-functionalized	25
	Carboxy-functionalized	-21
PBS, 1mM		
	Bare	-28
	Amine-functionalized	13
	Carboxy-functionalized	-23
PBS, 10mM		
	Bare	-21
	Amine-functionalized	9
	Carboxy-functionalized	-18
PBS, 100mM		
	Bare	-16
	Amine-functionalized	0
	Carboxy-functionalized	-11
pH (in DIW)		**Zeta potential (mV)**
Bare NP		
	5	-38
	7	-30
	10	-19
Amine-functionalized NP		
	5	37
	7	18
	10	5
Carboxyl-functionalized NP		
	5	-10
	7	-29
	10	-36

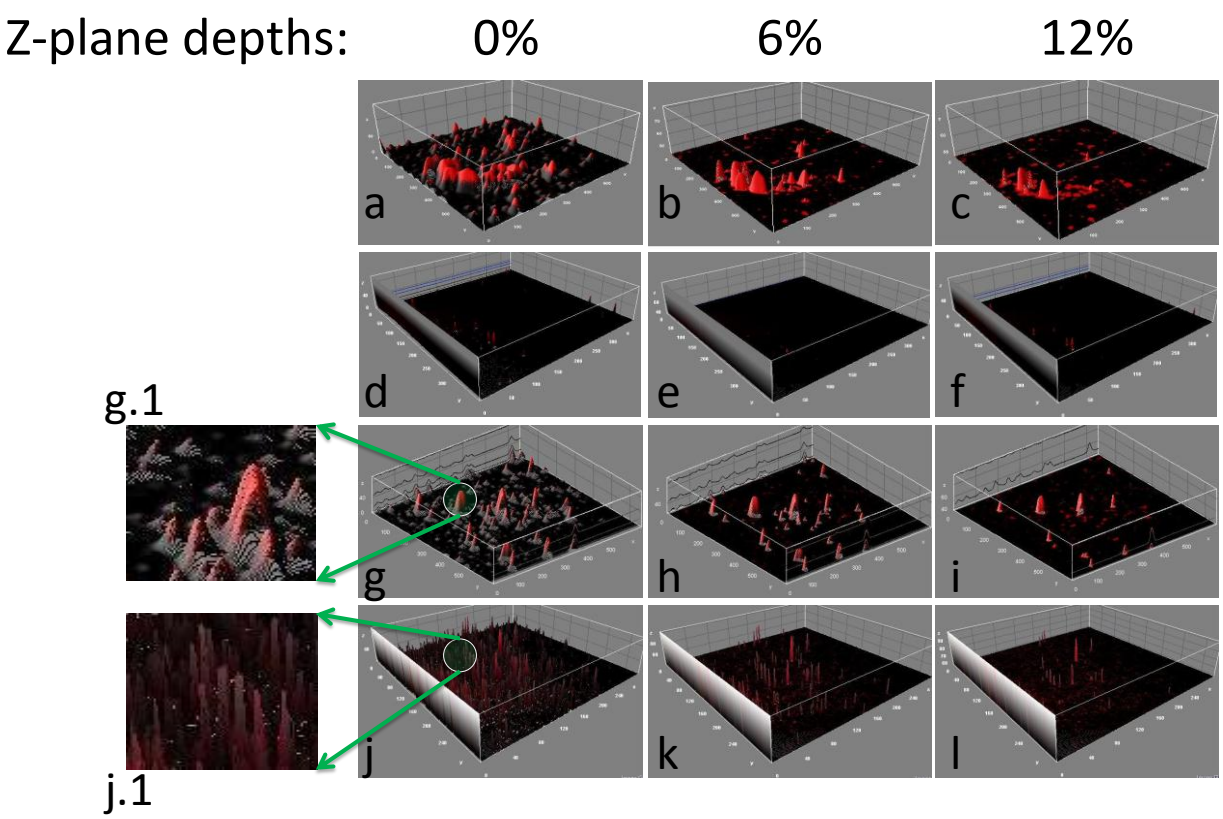


Supplementary Figure 1. Schematic representation of the proposed aggregation mechanism of the NPs in free and antibody-conjugated states. (A) Representation of bare and functionalized NPs, co-existing in in the solution as a mixed population of bare, partially functionalized and

completely functionalized NPs. (B) Depiction of a probable mechanism of aggregation of the silane-functionalized NPs by the non-covalent interaction, which is mainly reversible upon mild sonication. (C) Aggregation due to the covalent linkage between activated functional groups on the antibody/NP conjugate with the functional groups on other conjugates. This uncontrolled self/crosslinking results in aggregates which could not be separated upon sonication. Fluorescence microscopy images of anti-HRP conjugated amine and carboxy functionalized NP (without blocking) against HRP (D) and (E), which are controls for Figure 1.

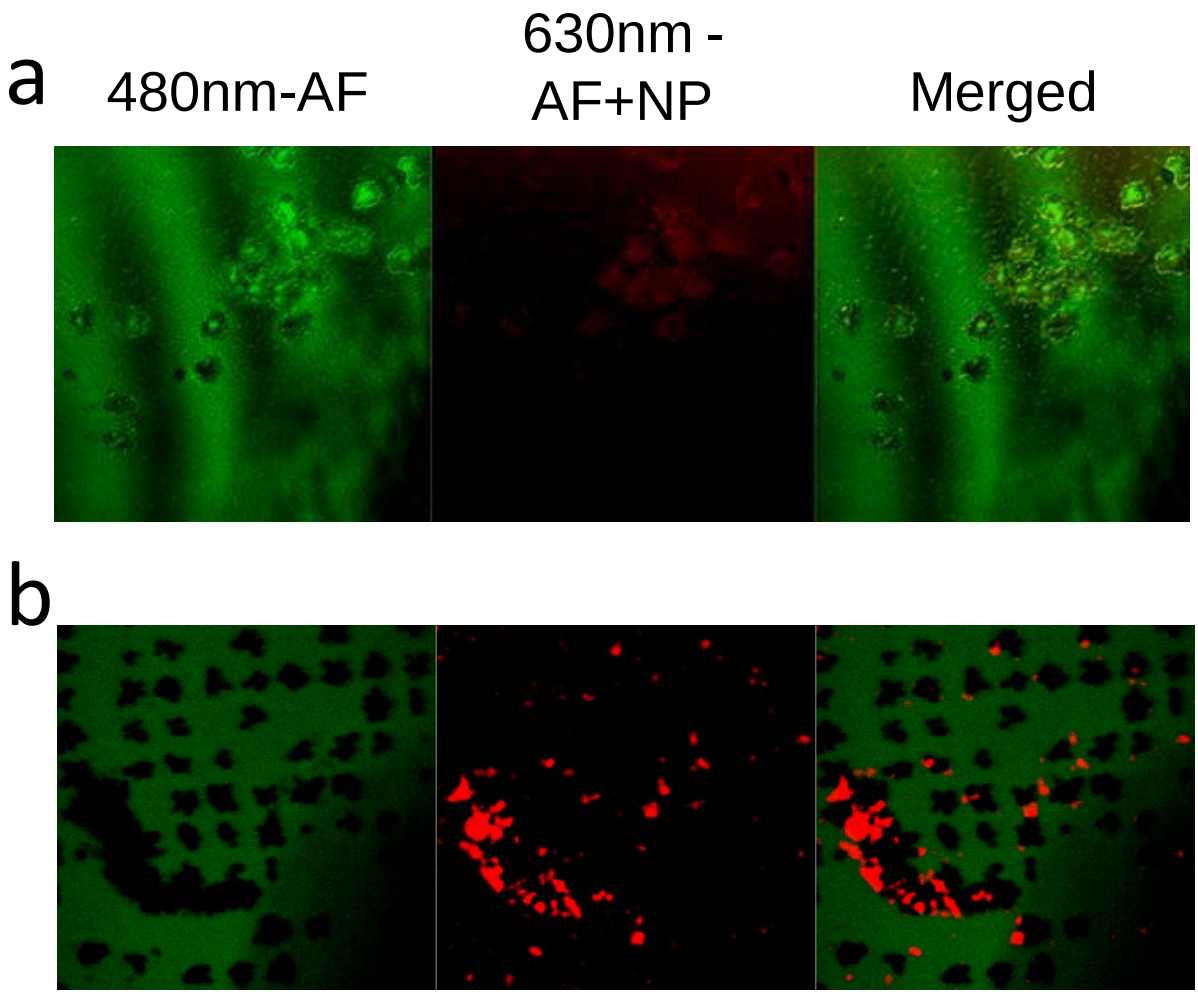


Supplementary Figure 2. Fluorescence imaging of the horseradish peroxidase (HRP) probing homogeneity and efficiency of the anti-HRP antibody-conjugated NPs. 'CfNP' and 'AfNP' are carboxy and amine-functionalized nanoparticles, respectively.



Supplementary Figure 3. Image J-based analysis of aggregate sizes in various NP-antibody conjugates (complementary to Figure 2 in main text and Supplementary Figure 1) as a function of thickness of the intensity-distribution peaks such that thickest peaks corresponds to big aggregates and thinnest peak represents smallest NP cluster distribution Panels from left to right illustrate the distribution of NP-antibody conjugates on basis of their sizes. This analysis is based upon the interactive 3D surface plotting plug-in function, which allows correlating qualitatively a 3D distribution profile of fluorescence intensity to the NP distribution and aggregation behaviour (depth allowance on Z-axis). In interactive 3D profiling the intensity is distributed over a given area (X=240units and Y=240 units) then by varying Z-axis the size of NP-antibody aggregates can be efficiently mapped as a function of thickness and number of peaks present in that area (e.g. panel j.1) and a comparison with respective controls (e.g. panel g.1) could be performed to analyze resolution of the big aggregates into fractions. This strategy allows visualizing peak-

thickness and based on that it could qualitatively be predicted that thick peaks represent aggregates while thin peaks correspond to smaller clumps with fewer NPs or may be clusters that are not aggregated. For example, in the case of panel ‘c’, for a default width allowance and depth allowance of 12% on Z-plane allowed us to map biggest aggregates of untreated AfNP-antibody conjugates. In the plane ‘b’, with a Z-plane depth allowance of 6% the intermediate size range of NP aggregates could be mapped. Whereas, in plane ‘a’ with a depth allowance of 0% all the smaller NP-antibody compositions were mapped. These Z-plane depth allowance settings were kept constant for all the panels from ‘a-l’. Panels a-c corresponds to anti-HRP antibody conjugated amine-functionalized NPs (AfNPs) that were not subjected to any treatment; whereas, d-f corresponds to AfNP conjugates that were subjected to the newly developed treatment strategy. Panels ‘g-i’ represent Carboxy functionalized NP (CfNP)-anti-HRP antibody conjugates without any treatment while ‘j-l’ are CfNP-antibody conjugates with appropriate treatment to reduce aggregation. It is evident from width-exclusion-based depth allowance analysis that the size of aggregates was significantly reduced after subjecting to the newly developed strategy for reducing cross-linking-mediated aggregation.



Supplementary Figure 4. (a) A double-negative control for the platelet probing using IgG (that was not specific to CD41 epitope)-conjugated carboxy-functionalized NPs that were not subjected to the novel treatment strategy reported in this manuscript. This control is to assess the non-specificity of the CfNPs toward surface. **(b)** A single negative control for the probing using anti-CD41 antibody-CfNP conjugates that were not subjected to the novel surface strategy as reported.