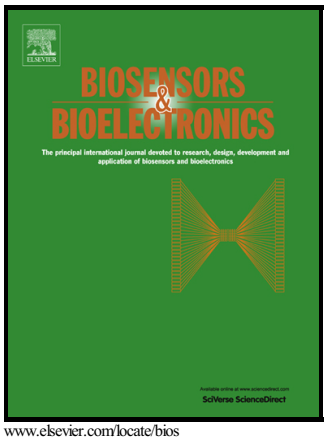

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PII: S0956-5663(14)00735-0
DOI: http://dx.doi.org/10.1016/j.bios.2014.09.048
Reference: BIOS7130

To appear in: *Biosensors and Bioelectronic*

Received date: 13 June 2014
Revised date: 28 August 2014
Accepted date: 22 September 2014

Cite this article as: KC Sanjaya, Andrea Ranzoni, Daniel Watterson, Paul Young and Matthew. A. Cooper, Evaluation of direct *versus* multi-layer passivation and capture chemistries for nanoparticle-based biosensor applications, *Biosensors and Bioelectronic,* http://dx.doi.org/10.1016/j.bios.2014.09.048

Evaluation of direct *versus* multi-layer passivation and capture chemistries for nanoparticle-based biosensor applications

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Abstract

Nanoparticles used in biosensor applications often fail when deployed directly in complex biological fluids. This is due to surface fouling and interference from the large concentration of non-specific binders (proteins, lipids, nucleic acids and saccharides) in the matrix. We systematically investigate four orthogonal approaches for decorating nanoparticle surfaces with affinity probes and evaluate their performance in buffer and serum. Carbodiimide coupling, cooper-mediated 'click' coupling, copper-free click coupling and thiol-maleimide coupling were quantitatively controlled during the fabrication process. Analyte mediated aggregation of fluorescent reporters and paramagnetic nanoparticle in a sandwich immunoassay was then used to probe assay sensitivity and specificity using an early biomarker of dengue fever, NS-1, as an exemplar and clinically relevant analyte. The type of surface functionalization played a vital role in assay performance in buffer vs. serum at the assay sensitivity limit (3 ng/mL in serum) and over the linearity of response of the assay's dynamic range. There was a 10 fold increase on the dynamic range of the detection of NS1 comparing copper free click coupling to carbodiimide coupling, one of the most common approaches for nanoparticle functionalization. By tuning their size, we could carefully monitor the evolution of nanoparticle populations by flow cytometer and discriminate between unbound and fluorescent nanoparticles. This subtle control on each assay component resulted in more than a 10-fold reduction in fluorescence background and improved the sensitivity of almost two orders of magnitude compared to endpoint measurements.

Keywords: surface chemistry, coupling chemistry, nanoparticle, paramagnetic, biosensor, Dengue, immunoassay.

1. Introduction

Surface engineering with biologically active molecules is essential in biosensing applications, where a target analyte is sequestered from a sample and each binding event then transduced into a signal. Capture of analytes can be mediated by affinity probes (Song et al. 2011), by conformational recognition (Liu and Lu 2006) or by coupling a specific nucleic acid sequence (Algar et al. 2011; Thomson and Cooper 2013) to a transducer. Bioactive micro- and nano-particles are very often used to probe the bulk of a fluid medium and accelerate the rate of capture of analyte using externally applied magnetic flux (Baudry et al. 2006). Nanoparticle surface functionalization with bioactive capture reagents can be achieved by direct coupling, through hydrophobic, electrostatic or covalent interactions (Mariagrazia Di Marco et al. 2010). Alternatively a surface molecular architecture, either embedded in the bioactive probe (Liu and Lu 2006; Thomson and Cooper 2013) or bound to an affinity probe (Song et al. 2011), can mediate the coupling. Introducing a linker structure in affinity-based biosensing enables more sophisticated surface molecular architectures (Cruz et al. 2011). In particular, functional moieties not present in biological molecules enable orthogonal chemistry coupling (Algar et al. 2011; Sletten and Bertozzi 2009), which ameliorates the risk of unwanted cross-linking, and also facilitates favorable orientation of the bioactive molecules. However, a literature survey reveals that two of the most common methods to immobilize antibodies on a nanoparticle surface still involve either carbodiimide chemistry (Smith et al. 2011; Song et al. 2011; Tsai et al. 2011) or the use of the high affinity interaction between streptavidin and biotin (Ran et al. 2014; Ranzoni et al. 2012). The latter is particularly suited for assembling customized capture probes on the nanoparticle surface and can be easily mediated by a linker (Ranzoni et al. 2012). However, the non-covalent nature of the interaction, despite its strength, limits its applicability for biosensing outside the laboratory (Norton et al. 1996; Ran et al. 2014). Carbodiimide chemistry results in non-oriented immobilization of capture probes (Hermanson 2013), potentially reduced functionality and bioavailability due to the lack of specificity of the linking method. Here large portions of the nanoparticle surface, which is often

hydrophobic, are exposed to the environment and represent potential sites for non-specific interactions.

Covalent linkage, either strongly electrostatic (e.g. DNA capture using layer-by-layer cationic or zwitterionic polymers), or effected by covalent coupling to hydrophilic polymers (e.g. dextran, polyethylene glycol), has been reported to help suppress the non-specific interactions deriving from endogenous interferents present in complex matrices such as human serum (Berry and Curtis 2003; Charles et al. 2009; Sperling and Parak 2010). Immediately after addition of particles to the biological sample (serum, plasma or other protein-rich matrix) a protein corona is formed on the surface of the particle. High molecular weight proteins bind to the surface of the particles unless these areas are blocked *prior* to addition to the biological matrix (Saptarshi et al. 2013). Undesired nanoparticle aggregation and occlusion of analyte-binding sites result in compromised assay performance, particularly with respect to sensitivity and dynamic range.

In this study we describe different molecular architectures built on the surface of nanoparticles. Direct immobilization of analyte-specific antibodies by means of a Carbodiimide cross linker (EDC) is chosen as reference control, due to its widespread use in nanoparticle-based diagnostics. Three different coupling chemistries are then used to covalently bind antibodies on a blocking layer of Human Serum Albumin (HSA). First, click-chemistry (Algar et al. 2011) was used to orthogonally couple the antibodies to the nanoparticle surface by means of short hydrophilic linkers (PEG8). Both copper-catalyzed cycloaddition (CuAAC) and copper-free reactions were tested using this linker. These approaches were contrasted with linking antibodies to the nanoparticle surface by means of a longer (5 kDa) linker, thus conferring more flexibility to capture agent. We also developed assays to carefully control and quantify each step, then assessed the impact of the adding linkers of progressive length with a fluorescent paramagnetic immunoassay for dengue virus non-structural protein NS1, an early biomarker of dengue fever (Fry et al. 2011; Peeling et al. 2010). We demonstrate dose-response curves for each of the developed molecular architectures, both in buffer and human serum.

2. Results and Discussion

Nine batches of nanoparticles were prepared according to the protocols described in the Supplementary Information. Each batch underwent a series of strict quality control steps to ensure minimal batch-to-batch variability (Table 1).

Table 1: Coupling chemistries and linking methods involved in the study

Particles Batch	Chemistry	Linker		#IgG/nm^2
		Particles	Antibodies	
NP_0_Amide	Carbodiimide	NA	NA	0.35 ± 0.02
NP_8_CuAAC	Cu Click	NHS-PEG-N_3	Alkyne-PEG-NHS	0.29 ± 0.01
NP_8_Cu Free	Cu Free Click	NHS-PEG-N_3	DBCO-PEG-NHS	0.36 ± 0.02
NP_5k_thioether	Thiol - Maleimide	NHS-PEG-SH	Sulfo-SMCC	0.14 ± 0.01

Colloidal superparamagnetic nanoparticles are widely used in diagnostics due to the large surface-to-volume ratio that enables fast capture kinetics of the target analyte directly in the fluid medium. Larger nanoparticles are generally very monodispersed and have high magnetic susceptibility, however the larger hydrodynamic ratio reflects in slower capture kinetics. Additionally, large hydrodynamic radius translates in a lower density of nanoparticles per unit volume. The target analytes distribute on the nanoparticle according to Poisson statistics, where the parameter of the distribution equals the average number of analytes per nanoparticle in solution (Ranzoni et al. 2012). Accordingly, multiple analytes can bind a single nanoparticle thus potentially decreasing the chances of both molecules to be available for completion of the sandwich (e.g. surface-based sensors (Dittmer et al. 2008) or cluster assay (Thomson and Cooper 2013). We have therefore selected sub-micron magnetic (500 nm) and fluorescent (200 nm) nanoparticles as optimal tradeoff to harvest both high magnetic susceptibility and high particle numbers in solution.

2.1 Particle blocking layer. Major aim of our research is direct deployment of the nanoparticles in human serum or plasma. Being the most abundant serum component and well known not to form aggregates, we have selected human serum albumin (HSA) to passivate the surface of our magnetic and fluorescent

nanoparticles. Titration of human serum albumin (HSA) (see Fig. 2A) enabled an optimization of the required amount of protein to achieve full coverage of the surface of the particles. Assuming that all particles are monodispersed, spherical and have negligible surface roughness, we have calculated the density of HSA on the nanoparticle surface. More than 3 molecules of HSA per nm^2 were immobilized on the nanoparticle surface for all the prepared batches of NP_8_CuAAC, NP_8_Cu Free and NP_5K_ thioether particles (See supplementary material). This value corresponds to several theoretical monolayers, calculated by finding the area of the mid-section of a sphere having radius equal to the Stoke radius of the protein (Cantarero et al. 1980). .However, we note that there is significant polydispersity in particle diameter (as determined by transmission electron microscopy; Fig. S8), which results in an overall increase in available surface area, compared to the theoretical value based on manufacturer's specifications. As a result, an increased surface area is available for the subsequent level of the surface architecture.

2.2 Bio-orthogonal linking chemistry. Hetero-functional linkers of different length and terminated at one end with a NHS-ester were anchored to the primary amines on the HSA blocking layer. Click-based architectures (NP_8_CuAAC and NP_8_Cu Free) were decorated with azide moieties on the second level of the architecture whereas NP_5K_ thioether are terminated with a sulfydryl group to enable antibody coupling (see Figure 1a). The available azide and sulfydryl groups react to alkyne, dibenzylcyclooctyne and maleimide groups respectively, which are introduced in a separate step on the affinity probes and are required to complete the conjugation reaction.

The degree of immobilization of the hetero-functional linkers was quantified with either a fluorescent (click-based architectures) or colorimetric assay (thioether architectures). In summary, dye molecules capable of binding the azide moieties have been reacted with a small sample from each nanoparticle batch. After performing a click reaction, unbound dye is removed and the fluorescence intensity emitted by the nanoparticles is correlated with the number of azide moieties on the surface by direct comparison with a standard curve. Similarly, a colorimetric substrate (5,5'-dithiobis-(2-nitrobenzoic acid)) is added to sulfydryl terminated nanoparticles. Such reagent generates a yellow response proportional to the number of sulfydryl groups in the sample. Direct correlation with a standard curve allows for

quantitatitative estimation of the number of linkers per nanoparticle. The molar excess of the linker was titrated (see Fig. 2C) until saturation was observed. A large excess (in comparison to the saturation value) ensured formation of a fully packed layer of linkers on the nanoparticle surface. The thick HSA base layer enabled anchoring of 0.58 linkers/nm^2, which in turn allowed calculation of a suitable molar excess of antibodies for the third layer of the surface architecture.

2.3 PEGylation of antibodies. Bio-orthogonal moieties were introduced on the antibodies by means of hetero-functional linkers complementary to the ones immobilized on the nanoparticle surface. This was the most delicate step in fabrication of the architecture since it could affect the activity and affinity of the antibody for analyte. The molar excess of the linker was optimized and, as shown in Fig. S5, we tested the effect of the presence of linkers by immobilizing NS1 protein on a surface and performing an ELISA. HRP-labeled anti-mouse secondary antibodies were used to quantify the number of PEGylated antibodies bound to the NS1 protein and no significant difference could be observed. Quantification of the amount of linker attached to the antibodies was vital. A large number of functional moieties on the antibody might result in unwanted cross-linking and nanoparticle aggregation. Additionally, the efficiency of the PEGylation reaction was strongly dependent on the particular antibody sequence, related to the number and distribution of –Lys and –Arg residues at the Fv region (data not shown). We could quantify the exact number of linkers introduced in the antibodies used for the copper free architecture. The dibenzylcyclooctyne (DBCO) has a characteristic absorbance spectrum with a peak at approximately 310 nm where antibodies have minimal absorbance. Consequently, the absorbance of the modified antibodies at 310 nm reveals the number of DBCO introduced in each antibody (see Supplementary Information). In our experiments, we introduce between 1 and 2 DBCO moieties per IgG.

2.4 Bio-functionalization of the nanoparticles. Linkage of the PEGylated antibodies to the molecular architecture on the nanoparticles is described in the Supplementary Information. In summary, antibodies were added in large excess compared to the estimated number of available linkers to favour generation of a densely packed monolayer. For this third, final layer, quantification of the immobilized antibodies could not be performed with a standard BCA assay due to

the high background deriving from the initial blocking HSA layer. Accordingly, we developed a surface ELISA where an anti-mouse fluorescent antibody bound to the capture probes on the nanoparticles. A different loading capacity for the different architectures was noted. The loading of antibodies is important in the assay as completely coated (saturated) particles are more stable and have better capture kinetics (Cantarero et al. 1980). From our calculations, one antibody molecule/nm^2 of particle surface represents a complete saturation and covers every possible parking area present on the particle surface.

For NP_0_Amide particles a carbodiimide cross linker was used to conjugate the antibodies to the particles. We estimated the amount of antibodies on the surface by means of a BCA assay and found 0.35 ± 0.02 antibodies were present per nm^2 of particle surface. Transmission electronic microscopy (TEM) images of the particles at different stages of conjugation are shown in Fig. S8.

2.5 Detection of NS1 antigen in using particle based sandwich assay

The performance of the developed architectures in was evaluated in two assay formats. First, a fluoroscent-magnetic sandwich immunoassay was used in which both capture and reporter antibody were bound to a magnetic, and a fluorescent nanoparticle, respectively (Thomson and Cooper 2013). This configuration has several advantages. Primarily, the same anti-fouling molecular architecture is mirrored on both solid phases. Additionally, the colloidal dispersion enables rapid capture and reaction kinetics as both binding components are free to diffuse in solution in three dimensions. We benchmarked the assay performance with a more standard assay protocol, similar to an ELISA configuration, where the capture antibody was immobilized on a solid surface and the surface architecture was limited to the fluorescent reporter.

Surface-based assays limit the possible readout methods to monitor fluorescence intensity to endpoint measurements, for example on a plate reader. However, such approach can only capture the total intensity emitted by every fluorescent label in solution. The particle-based assay enabled more flexibility in the readout technology. We have developed a method to discriminate all the nanoparticle populations by means of flow cytometer. In our assay, we selected magnetic nanoparticles with a diameter of 500 nm and fluorescent nanoparticles with a diameter of 200 nm.

Accordingly, the magnetic nanoparticles scatter abundant light in the forward and side direction, whereas fluorescent nanoparticles appear as a distinct population on a flow cytometer size plot. When size information was correlated with fluorescence intensity, bound fluorescent nanoparticles could be easily discriminated from free magnetic and free fluorescent nanoparticles, since they appeared as three independent populations in the FACS analysis. We plotted the percentage of gated events exhibiting side scattering and fluorescent intensity above a certain threshold and correlated this to the number of clusters (as outlined in the Supplementary Information). As a result, we could achieve a 10-fold reduction of the background fluorescence which resulted in two orders of magnitude lower limit of detection (See Supplementary Information).

Fig. 3 shows the dose-response curves in buffer and human serum for the different surface architectures. The carbodiimide cross linker gave very moderate sensitivity in buffer, and extremely poor performance in human serum, in which the high background signal when no antigen was present in solution resulted in an extremely high baseline noise level for the assay. Direct comparison between Fig.3b and 3c shows that the coupling chemistry employed has significant impact on the assay performance. In particular, NP_8_CuAAC shows the Hill slope to be 0.51, NP_8_Cu free shows the slope 1.01and NP_5k_thioether shows the slope 0.46. Remarkably, NP_8_Cu Free shows a much steeper slope in the linear part of the dose-response curve. This value shows that the slope is almost linear. Though the loading of antibodies was comparable between NP_8_CuAAC and NP_8_Cu free there was a remarkable effect on the assay performance. The use of a reducing agent (ascorbate) during the CUAAC reaction for immobilization of the PEGylated affinity probes on the azide modified nanoparticles might have contributed to the loss of function of the antibodies present. Previous literature has highlighted how the linker length requires empirical optimization (Cruz et al. 2011; Ranzoni et al. 2012). Theoretically, a longer linker should explore more efficiently the surrounding space, therefore accelerating the capture kinetics and facilitating favorable conformational alignment. Accordingly, we developed a PEG linker of 5000 g/mol on NP_5k_thioester. However, the anti-mouse assay showed the lowest loading of antibodies to the particles NP_5k_thioester. We investigated the origin of a reduced Hill Slope of MP_5k_thioester compared to MP_8_Cu_Free by performing a

'depletion study', where a known amount of NS1 is incubated with 5-fold excess of magnetic particles. After prolonged incubation, the non-captured NS1 remaining in the supernatant was estimated by ELISA (see Supplementary Information) and we observed a 1.2-fold lower binding capacity. We attribute this difference in capture efficiency as a tradeoff between lower antibody density on the surface of particle NP_5k_thioester but increased mobility of antibodies due to the presence of the long linker, which could lead to less steric hindrance and a better presentation to the solution phase to capture analyte. All linker-mediated architectures possessed a much lower background noise signal compared to the NP_0_Amide, resulting in dose-dependent responses over a wider dynamic range. Notably, the density of capture probes immobilized on the topmost layer of NP_8_CuFree and NP_0_Amide was almost identical.

We selected the best performing architecture for benchmarking on surface-based immunoassay. The Hill slope value for fluorescent ELISA was 0.44 on the linear region compared to 1.01 for magneto-fluorescent sandwich (see Supplementary information for details). Both antigen binding and fluorescent tagging are diffusion-limited processes and the lack of motility of immobilized antibodies results in lower capture kinetics. We therefore attribute the milder slopes and early saturation of the fluorescent signal to limited antigen bound on the surface. Additionally, the lack of protective layer induces a much higher background that effectively translates in poorer detection limit (LoD_{ELISA} = 1.05 ng/mL, LoD_{FACS} 0.15 ng/mL).

3. Conclusion

We have evaluated different surface molecular architectures for efficient immobilisation of affinity probes on an initial protein-based blocking layer. We have determined the impact of the different features of the architecture with a fluoro-magnetic assay with an early biomarker of dengue fever. Our assay achieved a lower limit of detection of 150 pg/mL in buffer and 3 ng/mL in human serum, one of the most sensitive immunoassays reported for this particular biomarker (Fry et al. 2011; Ganguly et al. 2014). We have demonstrated that simply decorating the nanoparticles with affinity probes does not translate into satisfactory assay performances (Saha et al. 2014) and more careful design to multi-layer linking strategies to passivate the surface and confer flexibility to the capture probes are

essential for efficient function in complex matrices such as serum. Our results demonstrate that blocking the particle surface is fundamental for specific, sensitive biosensing in biological fluids. The biochemical attachment strategy used affects assay performance; with orthogonal coupling chemistries (e.g. click chemistry) enabling not only precise cross-linking of the affinity probes, but also introduction of functional moieties that can be used to characterize and quality control each step of the reaction. Each of the proposed architectures could be readily assembled with commercially available reagents, and can therefore be deployed with great versatility on different substrates with minor modifications to the procedures described herein. Metallic electrodes or electrochemical biosensors (Fu et al. 2012) could benefit if molecular architectures with biofouling capabilities and densely pack affinity probes on the surface are employed. Surface-based immunoassays (e.g. ELISA) still rely on passive adsorption of capture reagents through non-specific hydrophobic and electrostatic interactions. Covalent linkage on passivated surfaces could considerably reduce the required sample preparation and blocking steps (Vashist et al. 2014). In summary, we believe that versatile strategies as the ones proposed in this Communication may find broad applicability in particle-based and surface-based biosensor assays.

Acknowledgements

The authors acknowledge the facilities, and the scientific and technical assistance, of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy and Microanalysis, The University of Queensland.

Figures

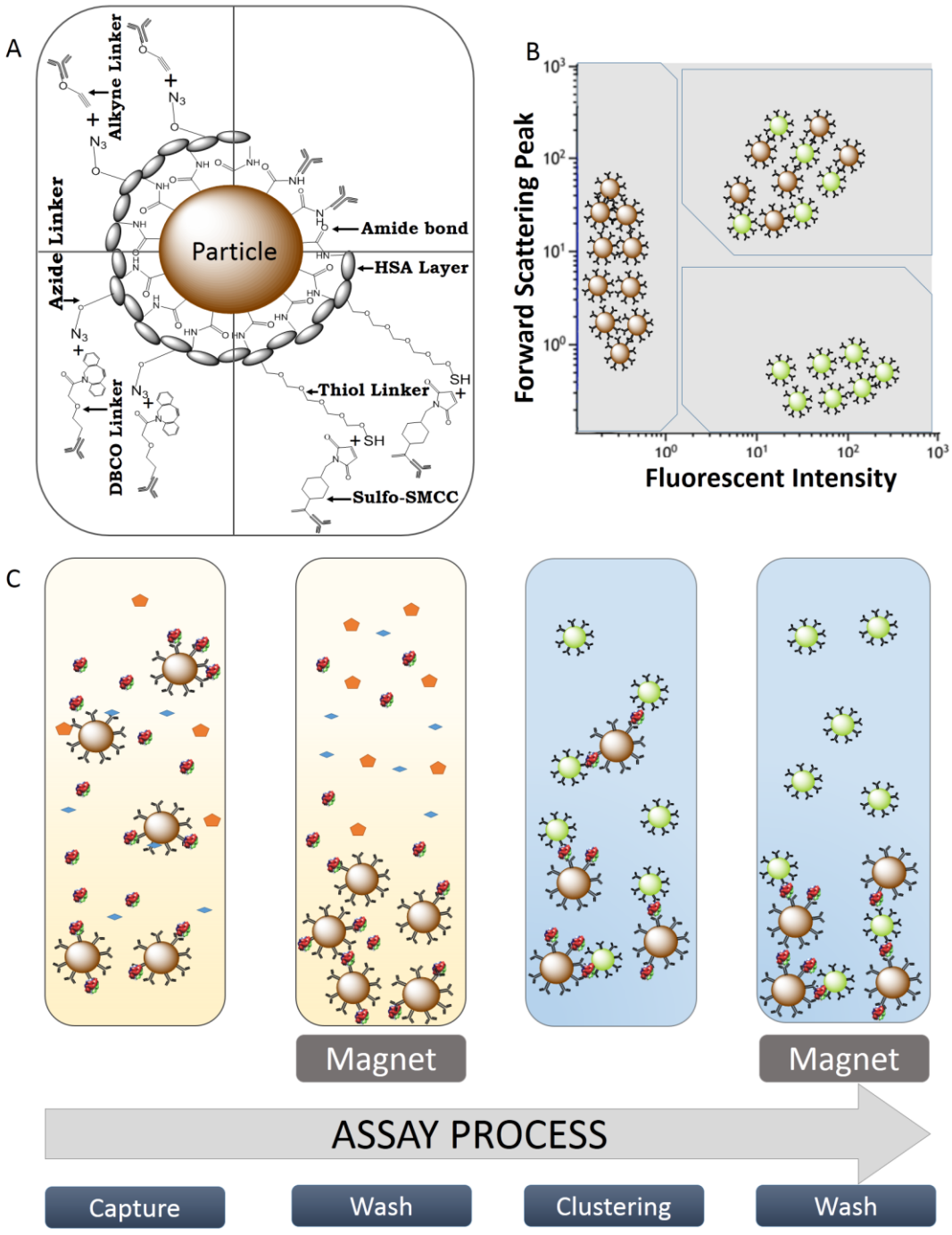

Figure 1: *A: Different surface architectures used to conjugate antibodies on particle surface, B. A typical FACS readout for cluster assay where fluro-magnetic clusters are seen at top right corner gated as MP_FP, C. Assay flow starting capture of NS1 in serum by magnetic particles followed by magnetic wash, addition of fluorescent particles and finally magnetic wash to remove any unbound fluorescent particles.*

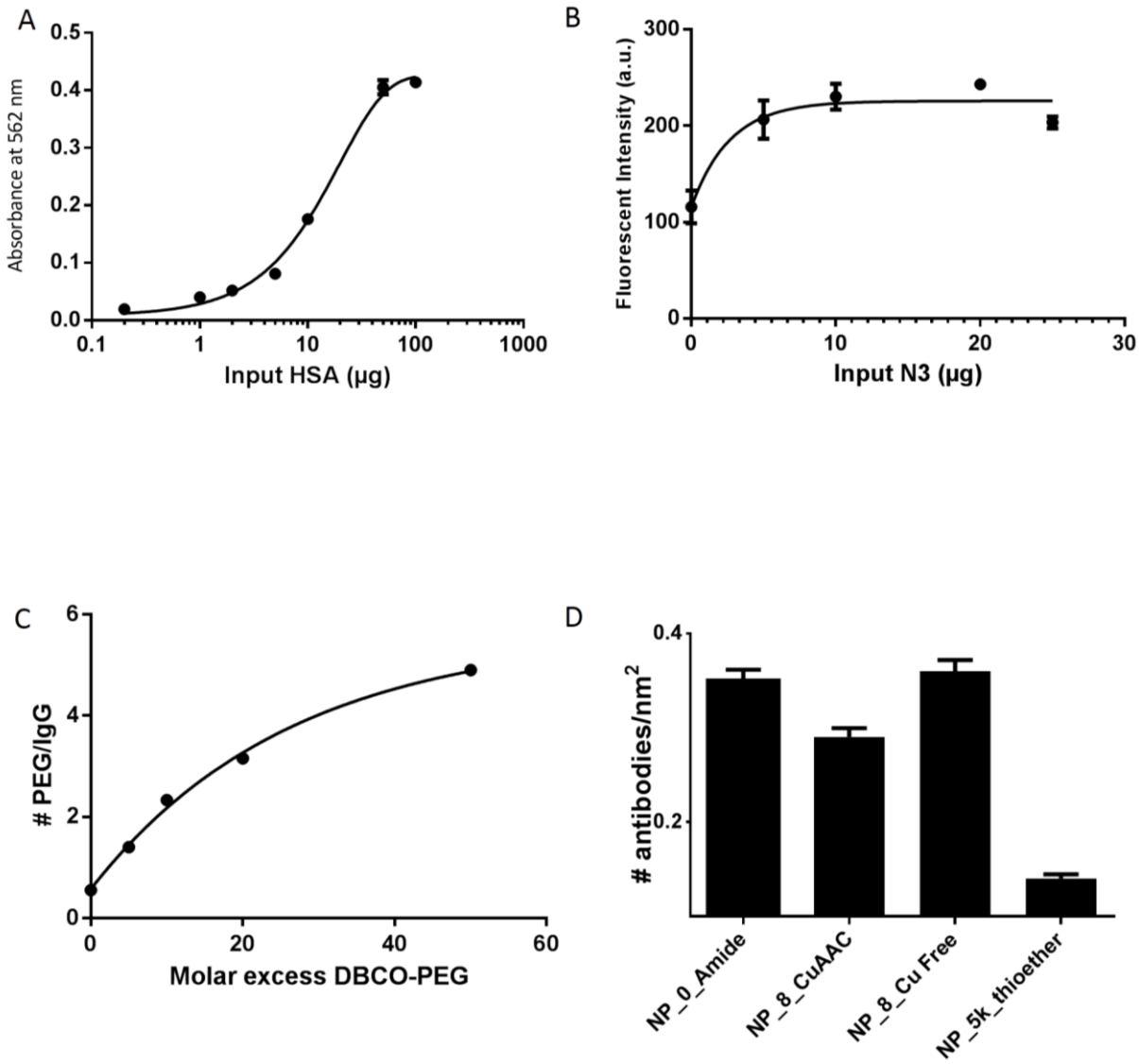

Figure 2: *A: Titration of HSA on the surface of particles to achieve a saturation, B: Titration of NHS-PEG linker on the particle surface to achieve full saturation of linkers on the particle, C: Titration of NHS-PEG linker to achieve at least 1 PEG linker per antibody, D: Different amount of antibodies present on the surface of particles.*

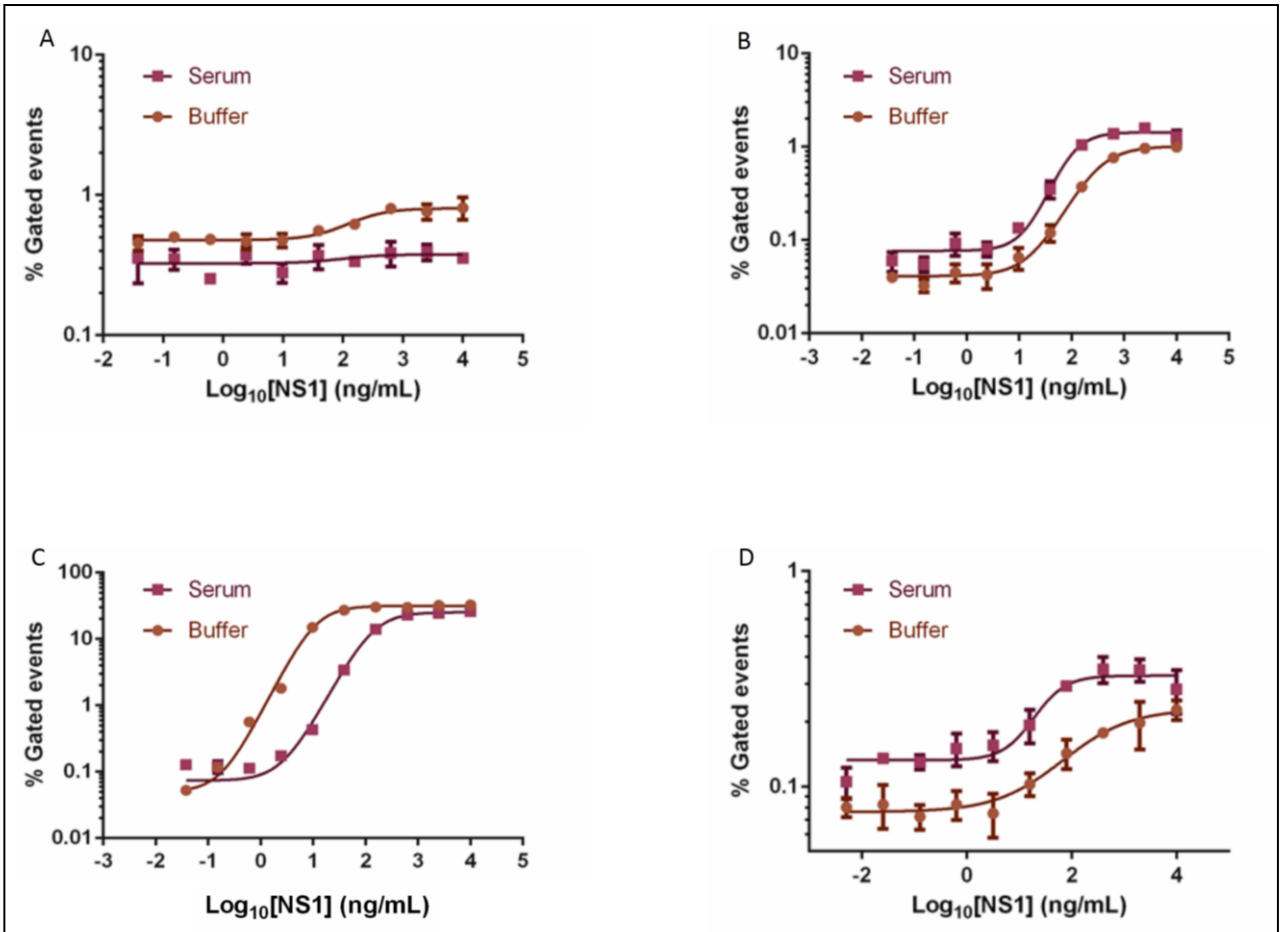

Figure 3: *A: Dose response curve for NP_0_ Amide, B: Dose response curve for NP_8_CuAAC, C: Dose response curve for NP_8_Cu free, D: Dose response curve for NP_5k_thioether*

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Highlights of work:

1. We demonstrated the dramatic impact for biosensing applications when adding a passivating layer on the surface of nanoparticles.
2. We achieved three novel bio-orthogonal conjugation strategies yielding different density of affinity probes on the nanoparticle surface and their impact on assay performances.
3. We developed a novel assay format based on fluorescent detection of nanoparticle clusters by means of flow-cytometer.
4. We highlighted the limitations of the current "gold standard", namely carbodiimide chemistry, when attempting direct deployment in complex biological samples.