Electronic Supplementary Material

Fluorescent Silver Nanoparticle based highly sensitive Immunoassay for early detection of HIV infection

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S1 Synthesis of Silver nanoparticles

The synthetic route that was chosen for synthesis of silver nanoparticles was chemical reduction where in a mild or strong reducing agent reduces the silver nitrate containing +1 oxidation state of silver to silver metal nanoparticles which contains the 0 oxidation state of silver. The reducing agent utilized for this process is hydrazine which is a mild reducing agent whereas glutathione is a capping agent which protects the silver nanoparticles from further aggregation thus maintaining the smaller size. Glutathione is a tripeptide containing glutamic acid, cysteine and glycine units and thus –SH (from cysteine), –COOH and $–NH_2$ are the active functional groups in the molecule. In presence of silver nitrate, the –SH from glutathione will interact with silver ions as the silver-thiol interaction is a very strong interaction and well established. Hence, the starting point of the reaction is the interaction between glutathione and silver nitrate which results in the formation of silver glutathionate.

$Ag^{+}_{(aq)} + GSH_{(aq)} \rightarrow [Ag(GSH)]^{+}_{(aq)}$

As the mixture of silver nitrate and glutathione is stirred, the pH of solution turns acidic and formation of a white supramolecular hydrogel is observed. This indicates the formation of $[Ag(GSH)]^{+}$ .The decrease in pH can be attributed to the release of H^{+} ions and $NO3^{-}$ ions upon interaction between –SH from glutathione and Ag^{+} from $AgNO_3$ respectively. Thus the presence of HNO_3 (H^{+} and NO_3^{-}) make the solution acidic. This is followed by addition of 1 M NaOH solution to make solution basic. At that stage, the mixture changes from a yellow colloidal state to completely transparent solution. The role played by NaOH is three fold (1)

1. Facilitates the dispersion of the $Ag[GSH]^{+}$ due to interaction with OH^{-} from NaOH.
2. The regulation of size and structure of the Ag[GSH] complexes formed after reduction.
3. It controls the reduction capacity of hydrazine which allows capping of the smaller formed Ag nanoparticles by glutathione.

The last step of the reaction is the reduction of Ag^{+} by Hydrazine to Ag^{0}. This step is allowed to continue for 10 hrs which allows size and structure refocussing of the nanoparticles thus ensuring that the nanoparticles grow to the optimum size and stability. (Refer: Highly luminescent silver nanoclusters with tunable emissions)

$$4Ag^{+} + NH_4 \rightarrow 4Ag^{0} + N_2 + 4H^{+}$$

S2 Computational modelling of bioconjugation process

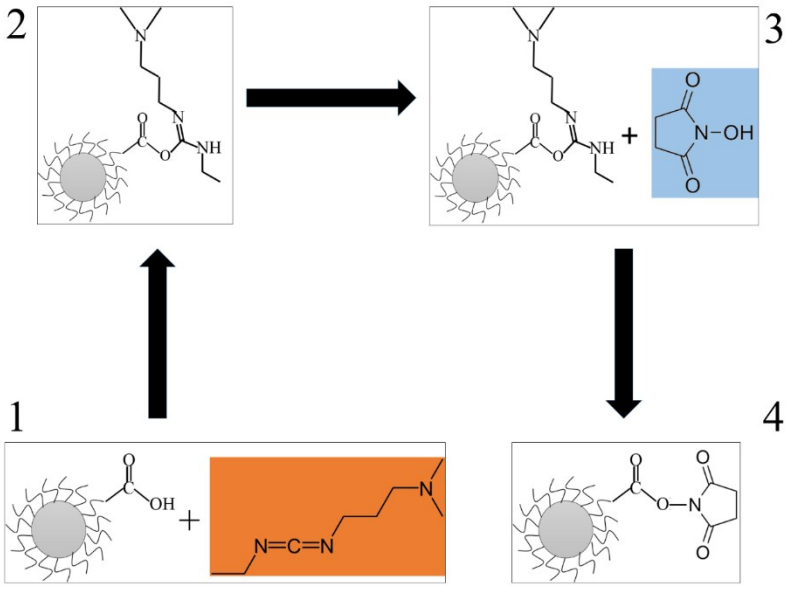
To simplify the study of this process, the coupling process was divided into four process stages as shown in scheme S-1 :

1) Reaction of Ag[GSH] with EDC

2) Formation of Ag[GSH]-EDC an unstable reaction intermediate

3) Reaction of the unstable intermediate with NHS

4) Formation for Ag[GSH]-NHS a stable product



Scheme S1: Schematic illustration of the four process steps involved in bio-conjugation

In order to understand the energetics of the individual process step, energies were calculated based on the energy of individual molecules tabulated in table S-1. It is evident from table S-2 that with

increase in the number of silver atoms the reaction is more effectively thermodynamically spontaneous. It is interesting to note the increase of energy in the second step which indicates the formation of the unstable o-Acylisourea intermediate that consequently interacts with NHS to form a stable product. This is confirmed by the juxtaposed energy profile of the net energy gain across the trend of increasing Ag atoms in the AgNPs cluster.

Table S-1: Optimized energies of all the molecules involved in the in silico studies.

Molecule	**Energy**
Glutathione	25.119
EDC	3.676
NHS	15.692
Glutathione-EDC	38.162
Glutathione-NHS	46.313
Ag-Glutathione	24.136
Ag-Glutathione-EDC	15.669
Ag-Glutathione-NHS	22.862
6Ag-Glutathione	24.577
6Ag-Glutathione-EDC	41.783
6Ag-Glutathione-NHS	23.054
31Ag-Glutathione	1002.35
31Ag-Glutathione-EDC	2774.845
31Ag-Glutathione-NHS	802.202

Table S-2: Energies of steps in the conjugation for systems with different number of Ag atoms

Process Step	**Energy (w/o Ag)**	**Energy (w/ Ag)**	**Energy (w/ 6Ag)**	**Energy (w/ 31Ag)**
Initial reactants	28.795	27.812	28.253	1006.026

Intermediary product	38.162	37.117	41.483	2774.845
Intermediary reactants	53.854	52.809	57.175	2790.537
Final product	46.313	22.862	23.054	802.202

While the magnitude of energy for each step is different for various Ag[GHS] system, the percentage change in energy can give an idea as to the efficiency of the ligand systems in comparison to each other. The difference in energies **ΔE** were calculated for the consecutive steps in the reaction for the four systems. It can be easily observed from table S-3 that the net energy gain is higher for the Ag[GSH] compared to the GSH . Additionally, the energy of the system in 31 Ag[GSH] seems higher compared to 6Ag[GSH] or Ag[GSH] in absolute energy terms. But when observed from the % energy point of view as depicted in Fig S-1, the change in energy is similar. Infact, % **ΔE** for 31 Ag system (40 %) is higher than the 6 Ag and 1 Ag system (both 38 %). We can conclude that the 31 atom system is more efficiently coupled to EDC-NHS than other Ag[GSH] systems

Table S-3: ΔE calculated for subsequent steps from the process diagram for the four different ligand systems

Process Step	ΔE_0	ΔE_1	ΔE_6	ΔE_{31}
Initial reactants	0	0	0	0
Intermediary product	9.367	9.305	13.23	1768.819
Intermediary reactants	25.059	24.977	28.922	1784.511
Final product	-7.541	-29.947	-34.121	-1988.34
Net energy Gain	-16.908	-39.252	-47.351	-3757.15

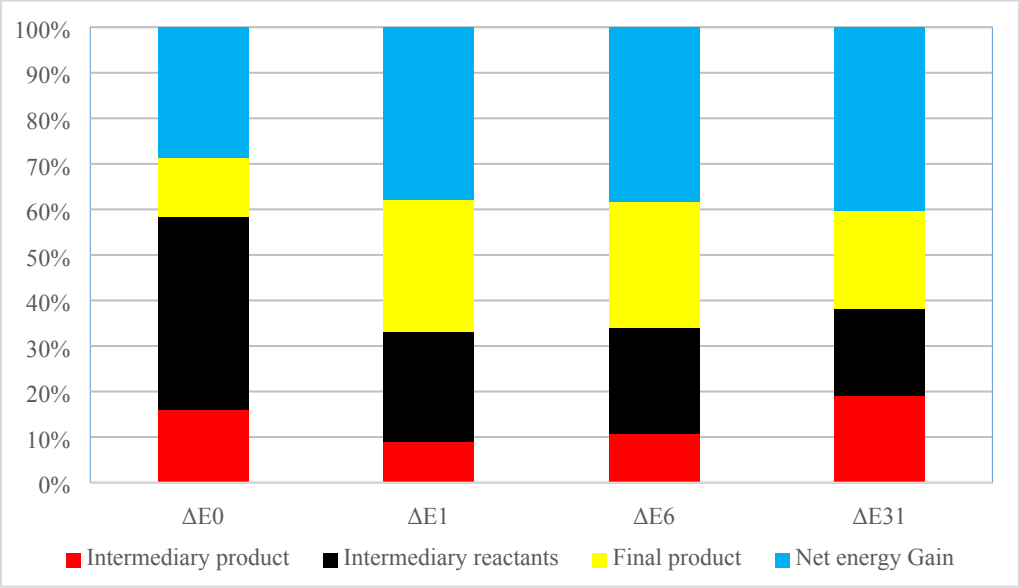


Fig S-1. Net energies for each process step plotted along with net energy gain

S3 Effect of bioconjugation on streptavidin biotin interaction

Based on the computational docking studies we tried to observe the effect of bioconjugation of streptavidin with FSNPs on streptavidin-biotin interaction. Based on the studies in MOE and HEX

we could easily see that there is a shift in the active site of biotin interaction on Streptavidin as seen in Fig S-2. Thus there is a structural rearrangement that occurs by bioconjugation which might cause the observed decrease in affinity for biotin interaction in case of biotin interaction.

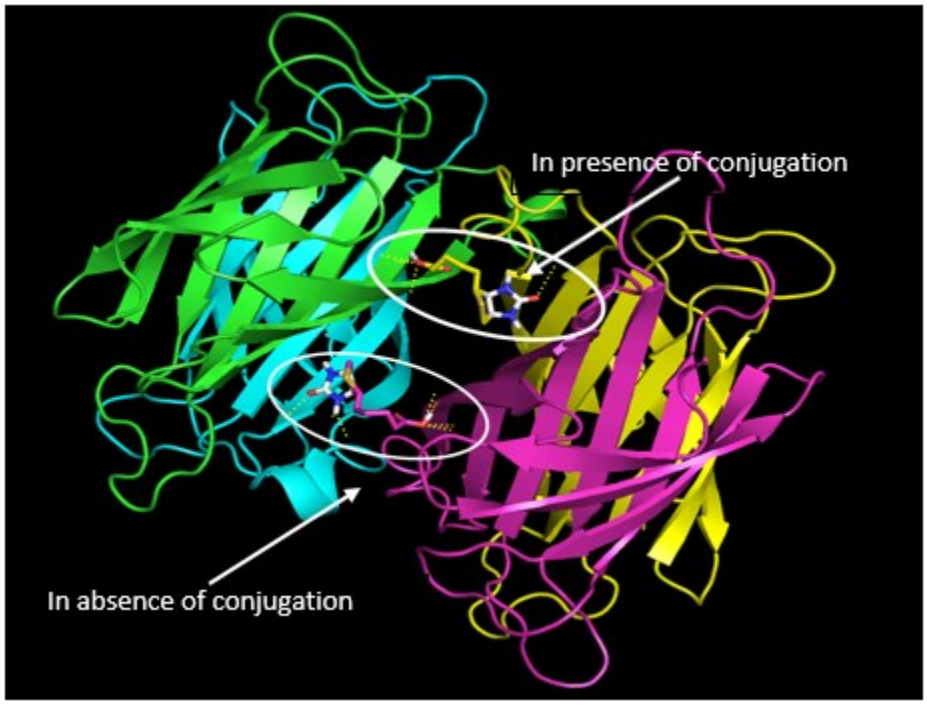


Fig S-2: The shift in the active site for biotin interaction in streptavidin in presence and absence of glutathione.

S-4 Components of Fluorescent Silver Nanoparticle Immunoassay

S-4.1 Format

The format of the assay was chosen to be sandwich immunoassay format. The Sandwich assay format, where the antigen is captured between two antibodies, is preferred because the assay becomes very sensitive and robust (up to 2 to 5 times more sensitive than direct or indirect)(2). Another advantage is that different reporting techniques can be used like chromogenic and fluorescence based for the same antigen(3).

S-4.2 Wash buffer

The coated plates are washed repeatedly with wash buffer, which in this protocol is, Tween-20 added to PBS buffer. Tween-20 which is a non-ionic detergent helps in removal of proteins which are loosely bound to plate surface in order to prevent the non-specific interactions(4). There are multiple wash steps throughout the immunoassay protocol, to ensure that all the unbound proteins are effectively removed.

S-4.3 Block buffer

The Casein Block Buffer (CBB) which is a protein based block buffer serves two purposes

1) Block the unoccupied sites on the microplate(5)
2) Stabilizing the capture antibodies which are bound to the plate surface by spacing them out and reducing the steric hindrance which could otherwise destabilize them(6).

The advantage of CBB is that being a protein based block buffer it needs to be added only once in the whole protocol as it is not easily washed away due to its amphipathic nature(7).

S-4.4 Antigen Sample

The purified HIV p24 is added is diluted in PBS wash buffer, the detergent present in the buffer disrupts the virions. The p24 is captured by monoclonal capture antibody when incubated for 1 hr at 37^{o}C. The 37^{o}C temperature was chosen as it reduces the incubation period to just 1 hr and also maintains precision in the assay from well to well(8). Following the incubation step the wells are washed five times to remove unbound p24 antigen to avoid their interference in the detection step.

S-4.5 Detector antibody

After the wash step, the detector antibody is added to the wells and incubated for 30 mins. The anti- p24 detector antibody is a biotinylated polyclonal antibody present in high-titer compared to p24 antigen(9). The incubation process allows the antibodies to interact with the antigen and

complete the antibody-antigen-antibody sandwich. The biotinylated antibodies play a key role in the detection step as they are the connecting biomolecules between the fluorescent probe which are streptavidin conjugated and the p24 antigen.

Comment [o]: Referencing style modified to Vancouver.

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