Supporting information

Plasmon enhanced fluoro-immunoassay using silver nanoparticles tagged protein as a novel blocking agent

Priyanka Sharma[!a,b], Manil Kukkar[!a], Ashok K. Ganguli[b] and C. Raman Suri*[a]

[a] CSIR-Institute of Microbial Technology, Sector 39-A, Chandigarh 160036, India
[b]Institute of Nanoscience and Technology, Sector 64, Mohali 160062, India

Molecular Alignment

The chemical structures of diuron and DCPU were energy minimized and the carbon C(1)-C(12); nitrogen N(1), N(4); chlorine Cl(13), Cl(14) and oxygen O(3) atoms were numbered respectively In addition, atoms C(2)-C(7), N(4), and C(8) of energy-minimized DCPU molecule were chosen to match with same atoms present in diuron by rms fit and overlay method.

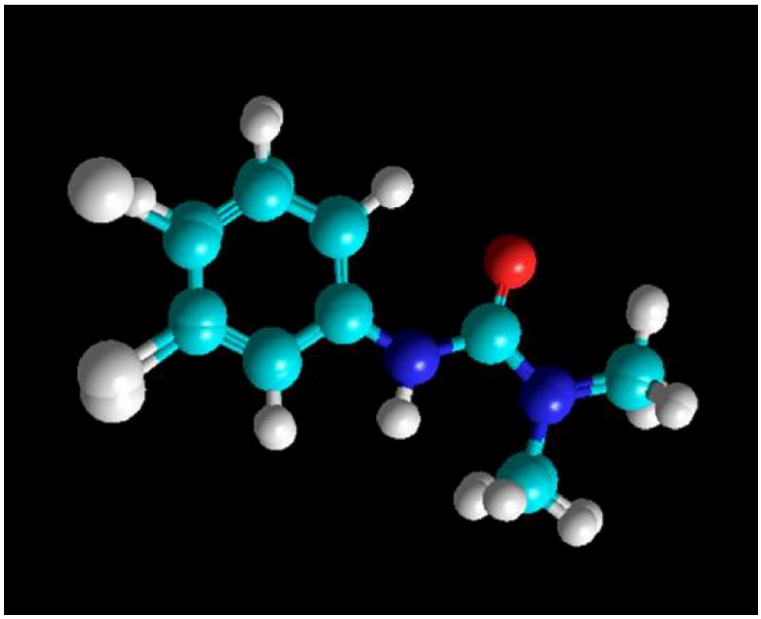

Fig. S1: Energy minimized (RMS fit) and overlay structures of diuron and DCPU using Hyperchem software.

Correlation analysis

In this study, it was observed that mass (M), refractivity (R), polarizability (P), surface area (SA), volume (V) and core interactions showed positive correlation with IC_{50}, as show below. However, hydration energy (HE), E_{HOMO}, E_{LUMO}, total energy (TE), binding energy (BE), electronic energy (EE), heat of formation (HF), isolated atomic energy (AE) and dipole moment (D) showed negative correlation. The positive correlated parameters are directly dependent, while the negative correlated parameters are inversely proportional to IC_{50} values. The predictive ability was cross-validated by the LOO method. The equation of the QSAR model was concluded as follows:

pIC50 = + 0.0719 (M) - 0.0505 (logP) + 0.0575 (R) - 0.0991 (HE) + 0.0636 (SA grid) + 0.0494 (SA app.) + 0.0621 (V) - 0.1024 (EHOMO) - 0.0813 (ELUMO) - 0.0796 (TE) - 0.0608 (BE) - 0.07 (EE) - 0.1035 (HF) + 0.0684 (CI) - 0.0805 (AE) - 0.0985(D)

	QSAR Descriptors	Correlation coefficient
1	Mass (amu)	0.96117249
2	Log P	-0.0084398
3	Refractivity ($Å^3$)	0.90080473
4	Polarizability ($Å^3$)	0.91267476
5	Hydration Energy (kcal/mol)	-0.6698356
6	Surface Area (A^{o2}) grid	0.93003487
7	Surface Area (A^{o2}) app.	0.85556052
8	Volume (A^{o3})	0.9231117
9	E_{HOMO} (eV)	-0.9583263
10	E_{LUMO} (eV)	-0.9847632
11	Total Energy (kcal/mol)	-0.9813396
12	Binding Energy (kcal/mol)	-0.9172074
13	Electronic Energy (kcal/mol)	-0.9546102
14	Heat of Formation (kcal/mol)	-0.9923821
15	Core Interactions (kcal/mol)	0.94893779
16	Isolated Atomic Energy (kcal/mol)	-0.9830641
17	Dipole moment (Debyes)	-0.5997444

Table T1: Various QSAR descriptors and their correlation with IC_{50}. The correlation analysis showed Pearson's coefficient correlation (r = 0.8857 with IC_{50}).

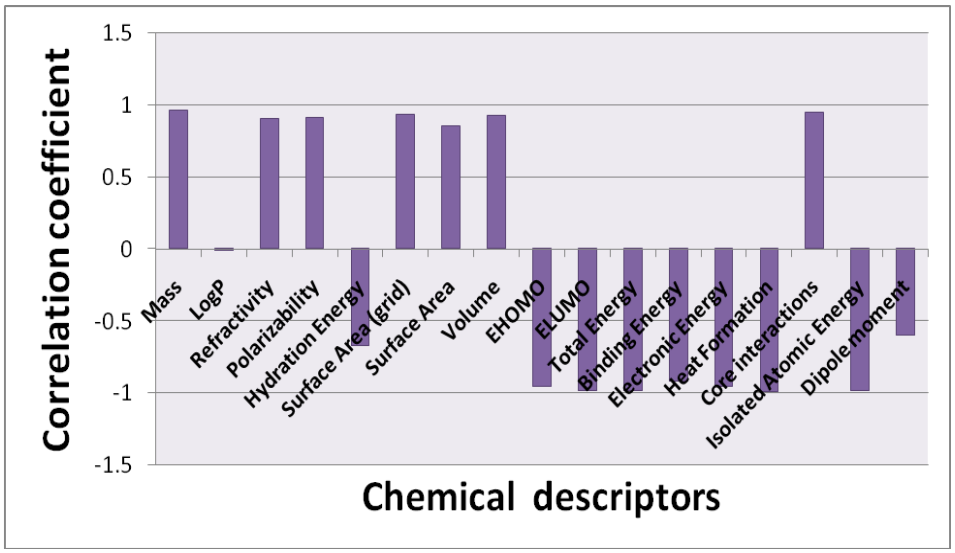

Fig. S2: Positive and negative correlation of chemical descriptors with IC50 (experimental)

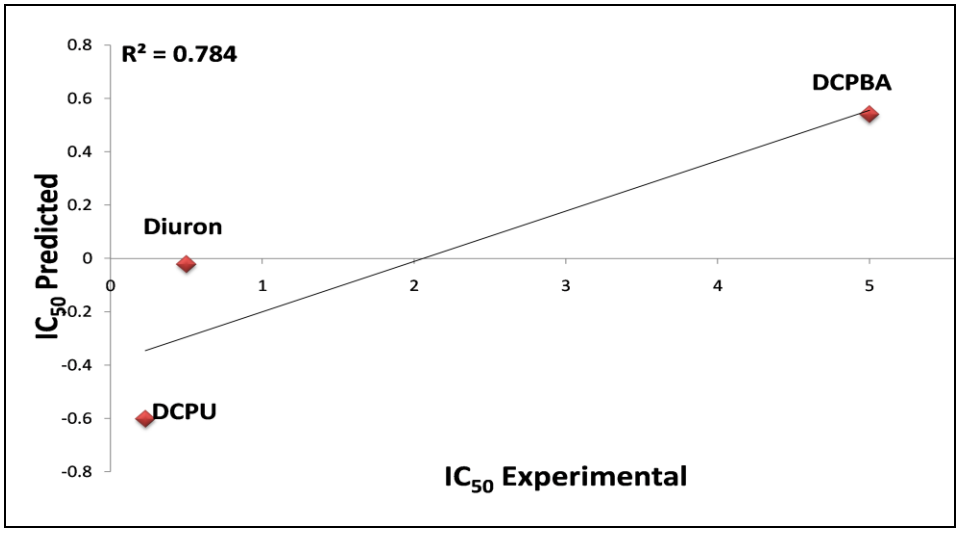

Fig. S3: 2D-QSAR model: experimental vs predicted IC50 values by SMO regression analysis.

Characterization of hapten protein conjugate using mass spectrometry

The hapten density of the conjugate, i.e., number of hapten molecules per protein molecule, was determined with a Voyager-DESTR mass spectrometer. Conjugate prepared at 1.5 mg/mL concentration was dialyzed against distilled water, mixed in equal amounts (1 µL each) with sinapinic acid solution (15 mg mL^{-1} in acetonitrile), and applied on a stainless steel probe with 0.5 µL of TFA solution (0.1%). The samples were allowed to dry at room temperature and then kept in the system for mass analysis. The spectra were acquired with 50 shots per sample in the linear mode at 30 kV and analyzed using the software provided with the system. Conjugation density for hapten resulted in a detectable increase in the molecular weight of the conjugate as determined by observing the peak shift of mass spectrum with reference to BSA (control).The molecular weight of the conjugate was calculated from the peak centroid of the peak. A change of ~4 KDa was observed (Fig. S4) indicating 20 hapten molecules are bound with BSA.

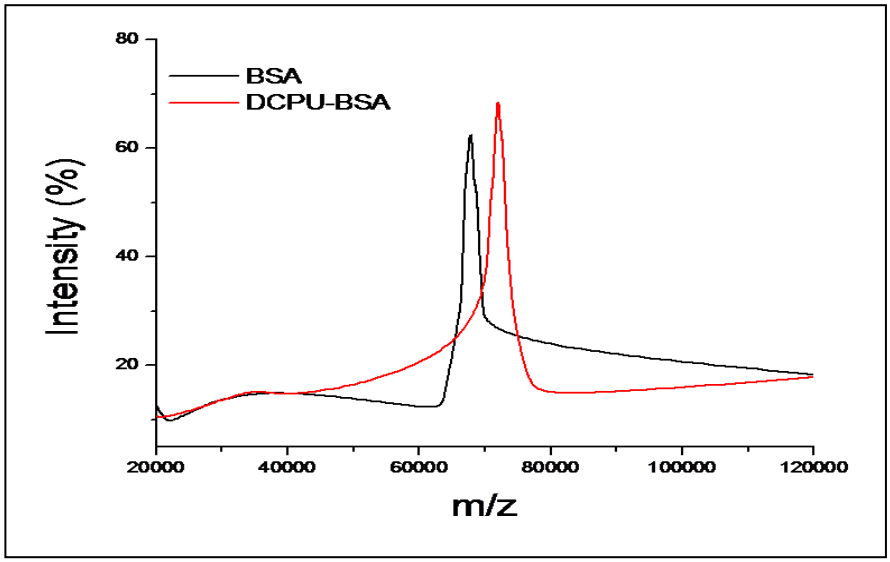

Fig. S4: Mass spectra showing shift in the mass of protein BSA when Hapten protein (DCPU-BSA) is formed.

Cross-reactivity of egg yolk anti-diuron antibodies with diuron and other analogues

The anti-diuron antibody was characterized by determining IC50 values for diuron and its major analogues by employing competitive ELISA as shown in the fig. S5 for the different concentrations (0.001 ng mL^{-1} to 10 µg mL^{-1}) of diuron, DCA, DCPMU and DCPU prepared in DDW.

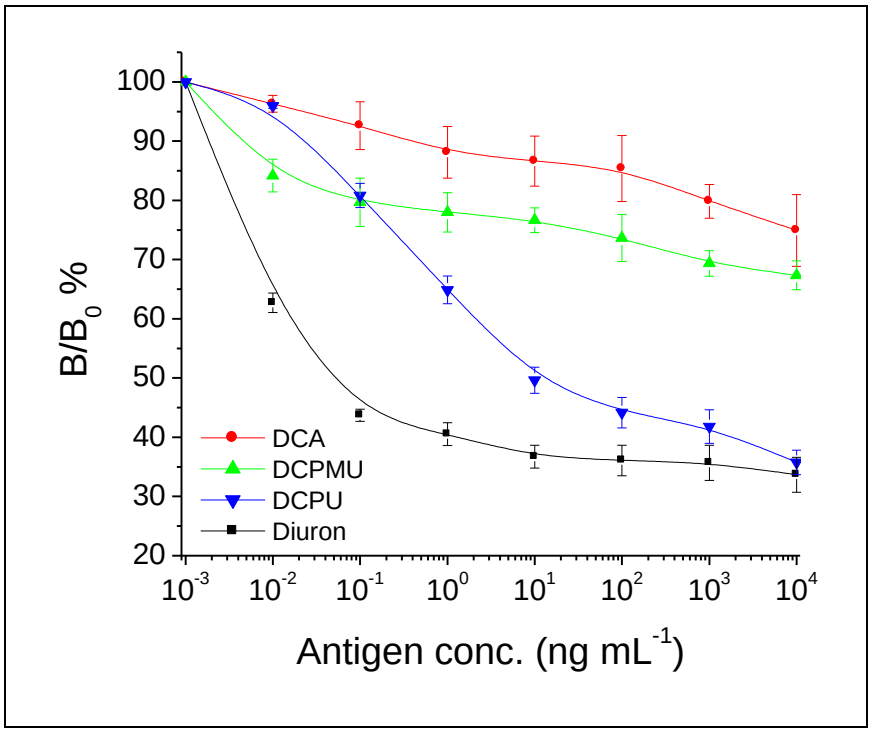

Fig. S5: Anti-diuron antibodies (IgY type) as characterized by competitive ELISA (n=3).

Stability studies of generated IgG and IgY antibodies

For comparative thermal kinetics studies of generated IgG and IgY types of antibodies, both antibodies were incubated at different temperatures viz. 30 °C, 37 °C, 45 °C, 60 °C, and the corresponding binding assay based on standard ELISA and CD spectra were taken at different time intervals viz. 0 h, 2 h, 4 h and 8 h respectively.

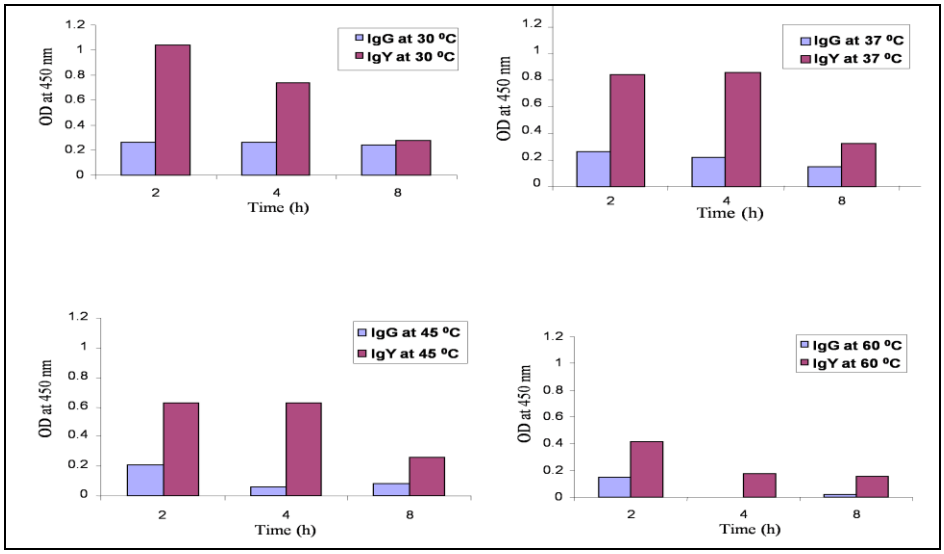

Fig. S6: Stability of IgG and IgY antibodies at different temperatures.

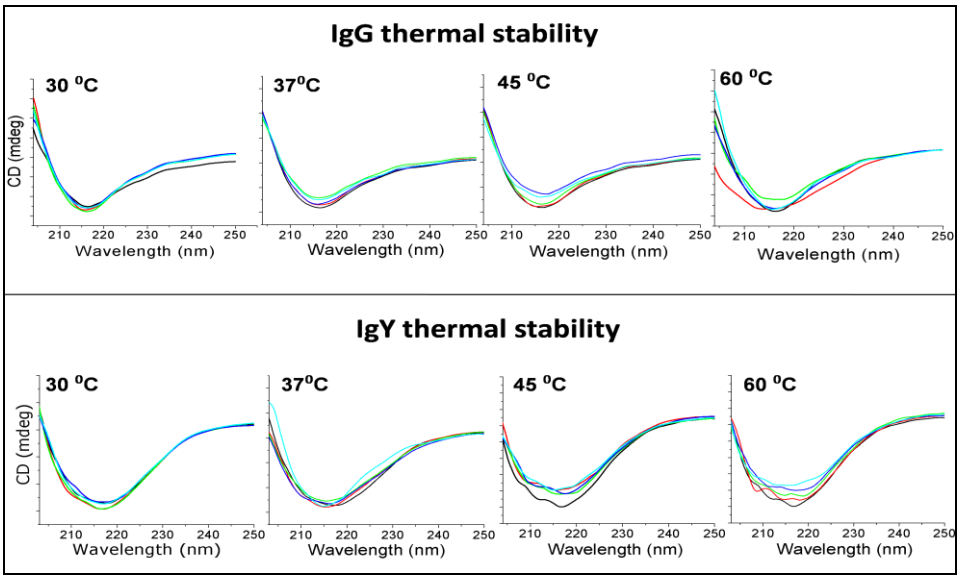

Fig. S7: CD spectroscopy of IgG and IgY anti-diuron antibodies respectively at different time intervals and temperatures .

Labeling of antibodies with FITC

Briefly, the antibody solution (2 mg mL^{-1}) was first dialyzed against carbonate buffer (50 mM, pH 9.2). A stock solution of FITC (1 mg mL^{-1}) was made in carbonate buffer and was used for preparing FITC labeled antibody conjugates (F:Ab). Different molar ratios of conjugates (C_1–C_5) were prepared by mixing different concentrations of FITC 100, 200, 300, 400 and 500 µg, respectively) with anti-diuron antibody (2 mg mL^{-1}) followed by an incubation for 2 h at room temperature. Conjugates were dialyzed against carbonate buffer for 24 h at 4 °C. Number of fluorophore molecules attached to antibody were calculated using the molar extinction coefficient of FITC-B (ε_0 = 70,000 $M^{-1}cm^{-1}$), the mean molar ratio of labeled fluorophore to antibody (F:Ab) was calculated from the equation (Thermo Scientific Tech Tips #6).

Moles dye per mole antibody (F:Ab) = A_{480nm} X dilution factor / ε0 × molar antibody concentration.

The concentration of antibody was calculated by dividing the absorbance value at 280 nm with the extinction coefficient (ε) of antibody (IgY) equal to 1.5 mg mL^{-1}

Protein Concentration (M) = $[A_{280} - (A_{max}$ x Correction Factor) / ε Protein] x Dilution Factor

Conjugates	A_{280}	A_{max}	Correction Factor (CF)
C_1	1.19	1.072	1.11
C_2	1.65	1.350	1.2
C_3	2.405	2.325	1.03
C_4	2.5	2.319	1.14

Table S2: The absorption at 280 and the absorption maxima values from the antibody:FITC conjugates (C_1-C_4) and their respective correction factors.

Morphological characterization of SNPs by TEM

TEM micrographs confirmed the size of the synthesized GNPs using optimum concentration of citrate as reducing agent was ~30 nm.

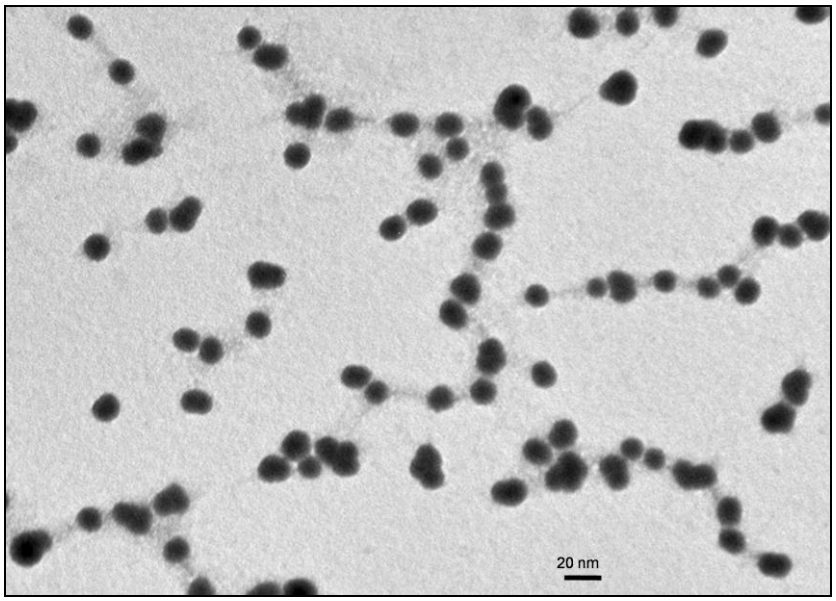

Fig. S9: TEM micrograph showing morphological details of silver nanoparticles

SNPs tagging with BSA: Flocculation assay

The minimum amount of protein required for optimum tagging with SNPs was optimized by employing a flocculation assay. In this assay, serial dilutions of both the protein solutions (BSA) were prepared. 100 µL of each dilution was added to 1 ml of SNP solution. After 15 min, flocculation was induced by adding 100 µL of 10% NaCl and absorbance scan was measured from 225-600 nm. The amount of protein necessary to prevent flocculation was deduced graphically from the concentration at which the absorbance becomes nearly constant. The initial concentration of the protein was checked using UV–vis spectrophotometer by measuring the absorbance of protein solution at 280 nm. SNPs solution was mixed with protein solutions of different concentrations and the mixture was incubated under mild shaking for 2 h at room temperature. After incubation, the mixture was centrifuged at 12,000 rpm for 30 min and washed (3x) with deionized water and the absorbance of the supernatant was measured at 280 nm. The amount of protein bound to the SNPs was determined by subtracting the free protein in the supernatant from the total amount of the protein added initially.

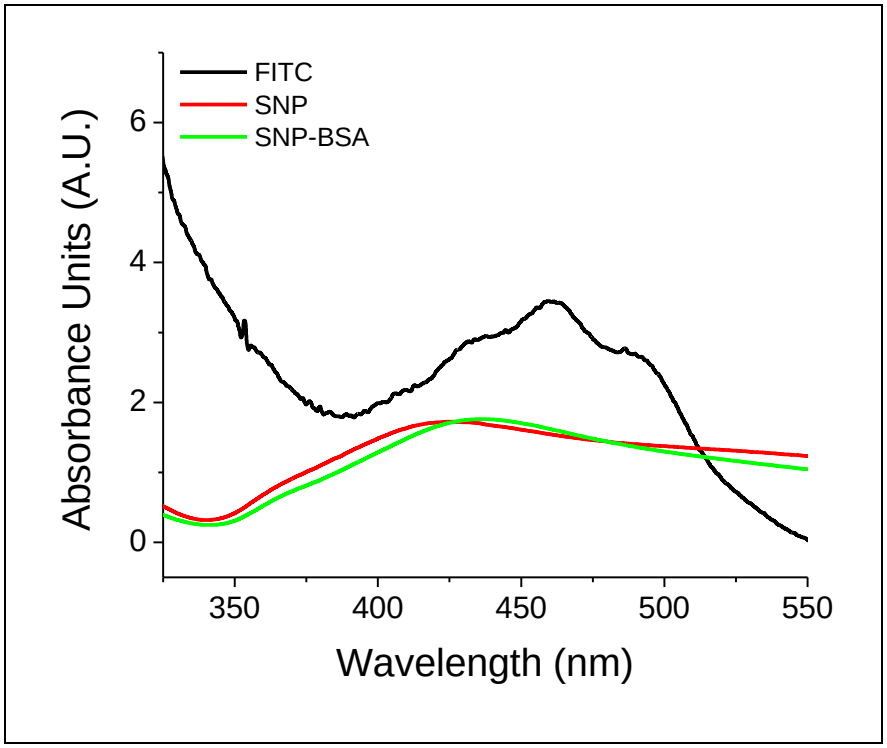

Fig. S9: UV-Vis absorbance spectra of FITC (black line); SNPs (red line) and SNPs functionalized with antibody (green line)

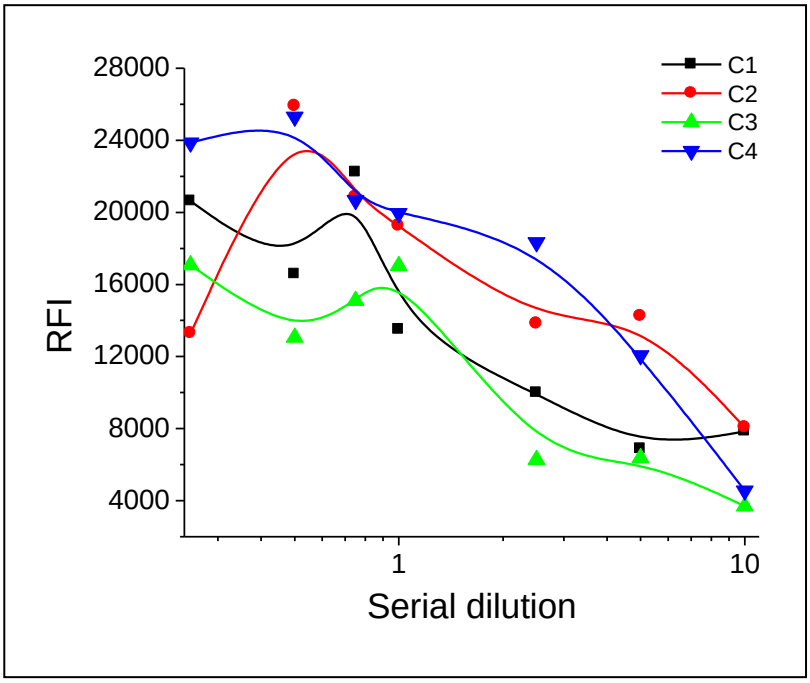

Fig. S10: Binding studies of anti-diuron antibodies labeled with FITC molecules at different molar ratios (C_1–C_4 respectively) using siver nanoparticles (SNPs) as such on microtiter wells.