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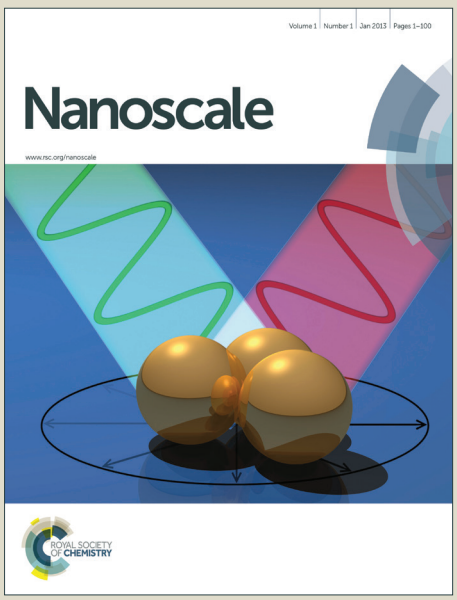

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Plasmonic ELISA for the detection of gp120 at ultralow concentrations with the naked eye†

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The technique of plasmonic ELISA is utilised here to detect the HIV-1 protein gp120 with the ultralow limit of detection of $8 \cdot 10^{-20}$ M (10^{-17} g·mL^{-1}) by an independent laboratory. It was corroborated that changes in the concentration of hydrogen peroxide as small as 0.05 μM could lead to nanoparticle solutions of completely different tonality.

The demand of ultrasensitive detection systems is rapidly increasing in applications such as cancer diagnosis,[1] the detection of pathogens[2] or the detection of toxic pollutants.[3] In recent years a new generation of biosensors has been proposed that utilises nanoparticle growth processes as the signal generation step of ultrasensitive detection systems.[4,5] When coupled to the high specificity and selectivity of antibodies, these methods can detect target molecules such as prostate specific antigen (PSA) and antigen p24 with a limit of detection (LOD) as low as $4 \cdot 10^{-20}$ M (10^{-18} g·mL^{-1}). [4,5] Viral proteins such as gp120 are excellent targets for ultrasensitive detection systems because they are not present in the proteome of the patient, and therefore their detection at ultralow concentrations could potentially be applied for the early detection of viral infection. Plasmonic ELISA offers the benefits of the well-established ELISA protocol for detection, blocking and washing steps. [4] In this technique, the key step to detect analytes at ultralow concentrations is to tune the kinetics of growth of gold nanoparticles growing in the presence of 2-(N-morpholino)ethanesulfonic acid (MES) in order to obtain either blue-coloured or red-coloured nanoparticle solutions. This is achieved by controlling the concentration of hydrogen peroxide in the growth solution with catalase, which is the enzyme label of the ELISA assay. The reaction was designed so that the outcome of the nanoparticle growth process is extremely sensitive to the concentration of hydrogen peroxide in solution.[6] However, this outcome also depends on other factors typically affecting nanoparticle growth processes such as the age of the solutions containing nanoparticle growth precursors, the reaction scale, the type of vessel where the reaction is taking place, and the presence of convection, among others.[6] Moreover, different individuals can introduce subtle variations into the growth process, for example, when mixing solutions (convection), which can affect the outcome of the nanoparticle growth step. Here, we demonstrate that plasmonic ELISA can detect the HIV-related protein gp120 at the ultralow concentration of $8 \cdot 10^{-20}$ M (10^{-17} g·mL^{-1}) when the reaction conditions are well controlled and a different individual performs the whole process in an independent laboratory.

Methods

A detailed protocol of plasmonic ELISA has been published elsewhere.[6] The detection of gp120 proceeded as follows. Nunc MaxiSorp® flat-bottom 96 well plates were modified with 100 μL of goat anti-rat IgG (Sigma, 1:100 in PBS) overnight. The plates were washed three times with PBS containing 0.1% Tween (PBST). Then, the plates were blocked with 300 μL of PBS containing 1 mg·mL^{-1} BSA (PBS-BSA). After washing the plates three times with PBST, 100 μL of monoclonal anti-gp120 developed in rat (diluted 1:100 with PBS-BSA) were added. In the mean time, a solution containing 10^{-4} g·mL^{-1} gp120 was serially diluted in fetal bovine serum (FBS) to obtain solutions with the concentration of 10^{-18} g·mL^{-1} or higher. The same dilution series was also performed with BSA to check for the presence of non-specific interactions that could lead to high noise levels. After 1 h, the plates were washed three times with PBST and 100 μL of the solutions containing either gp120 or BSA at different concentrations were added to the plate for 2 h. Subsequently the plates were washed five times with PBST and modified with 100 μL of polyclonal anti-gp120 developed in sheep (diluted 1:100 in PBS-BSA). After 1 h the plates were washed three times with PBST and modified with 100 μL of biotinylated anti-sheep IgG (Abcam, diluted 1:300 in PBS-BSA) for 1 h. Then the plates were washed three times with PBST and modified with 100 μL of a streptavidin-catalase conjugate diluted 1:50 in PBS-BSA. The preparation of the conjugate has been previously described.[6]

After 30 min, the plates were washed five times with PBST, three times with PBS and two times with water. The plates were then dried and 100 μL of a 240 μM H_2O_2 solution in 1 mM MES buffer pH ca. 6.5 were added for 30 min. The 1 mM MES buffer was obtained by diluting a stock solution with the concentration of 0.1 M (pH 6.5). Then, 100 μL of freshly prepared 0.2 mM gold (III) chloride trihydrate solution in 1 mM MES buffer were added. This solution was obtained by diluting a 0.1 M stock solution in water with 1 mM MES buffer pH ca. 6.5. The nanoparticle growth process was stopped when the control experiments acquired a red tonality by adding 50 μL of 100 μM glutathione to each well.[7] The absorbance at 550 nm was measured with a V-670 Jasco spectrophotometer.

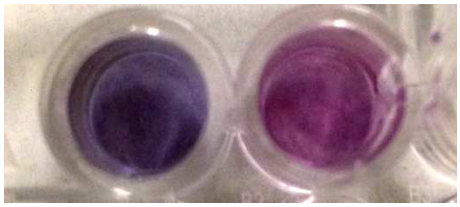

Figure 1. Nanoparticle solutions grown in 1 mM MES buffer (pH ca. 6.5) and with hydrogen peroxide at a concentration of 119.95 μM (left) and 120 μM (right). The final gold (III) chloride concentration was 0.1 mM.

Results and discussion

The effect of extremely small changes in the concentration of H_2O_2 on the nanoparticle growth process was studied by growing nanoparticles with solutions of different H_2O_2 concentration. Figure 1 shows that the solution containing H_2O_2 with the final concentration of 119.95 μM is clearly blue whereas the solution containing H_2O_2 with the final concentration of 120 μM is undoubtedly red. These results demonstrate that the generation of nanoparticle solutions with characteristic tonality can be achieved with extremely small changes in the concentration of hydrogen peroxide, as previously observed.[6] The tonality of the solution is so strikingly different that it is reasonable to think that changes in tonality could be achieved with even smaller changes in the concentration of hydrogen peroxide.

It should be noted that, after some time, both nanoparticle solutions acquire a red tonality unless the nanoparticle growth process is stopped, for example by adding 20 μM glutathione.[7] This observation fits well with the idea that the nanoparticle growth process is kinetically controlled, as previously observed in many other nanoparticle growth processes.[8-10] Indeed, the reaction takes place in an excess of MES buffer, which is also a weak reducing agent. Therefore the role of the hydrogen peroxide is not to stoichiometrically reduce gold ions but to tune the kinetics of growth in order to yield nanoparticles with different morphology and consequently different optical properties. Consequently, it is not necessary to prepare H_2O_2 with the exact concentration 120.00 μM in order to achieve the reported results as the observed differences are time-dependent and would yield the same result within a certain concentration range, the only difference being that the growth time period may be slightly different. Similarly, the abrupt colour change reported here does not contradict the previously published data in which colour changes where observed with large increments of H_2O_2 (Fig.2 in reference 4) because the colour of the solution does not only depend on the concentration of H_2O_2 but also on the growth time. Furthermore, although the exact mechanism behind this extremely sensitive nanoparticle growth process is under investigation, it should be noted that there are some precedents of nanoparticle growth processes whose outcome is extremely dependent on small variations of the reaction conditions. For example, it is well known that the growth of gold nanorods is extremely dependent on the presence of impurities in the precursors of the reactions and that reagents of high purity purchased from different companies, or from the same company but from different lots, can yield nanoparticles with different morphology, and hence with different optical properties.[11-12]

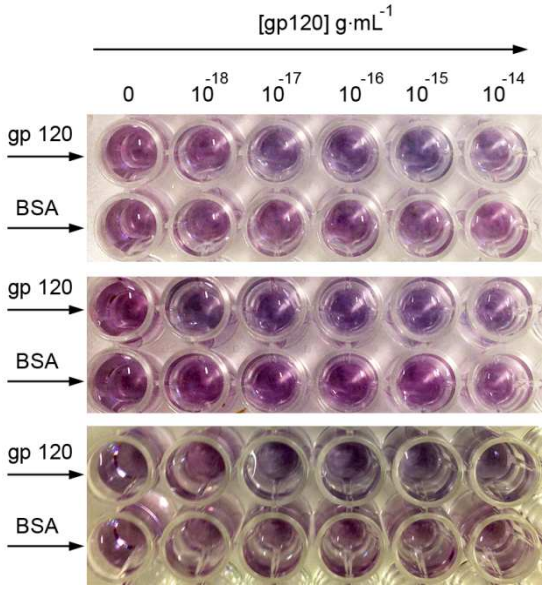

Figure 2. Naked-eye detection of gp120 with plasmonic ELISA

Figure 2 shows the naked-eye detection of gp120 spiked into FBS. The LOD defined as the lowest concentration of gp120 that yields blue-coloured nanoparticle solutions in three different experiments is 10^{-17} g·mL^{-1} ($8{\cdot}10^{-20}$ M). 18 control experiments performed in exactly the same conditions but diluting an unrelated molecule (BSA) yielded red-coloured nanoparticle solutions in all instances. Red-coloured nanoparticle solutions were also obtained in all the experiments in the absence of gp120 ("0" in Fig. 2). In Figure 3, the LOD defined as the concentration of analyte that yields a signal higher than two times the standard deviation of the blank is 10^{-17} g·mL^{-1} ($8{\cdot}10^{-20}$ M) (σ_{blank} = 0.1). It should be noted that the solutions for calibrating the system were obtained by serially diluting a stock solution containing gp120 with the concentration of 10^{-4} g·mL^{-1}. The pipettes used herein (ThermoScientific Finnpipette^{TM}) have according to the manufacturer an inaccuracy (systematic error) of 1% for the 100μl and 0.6% for the 1000μl pipettes and random errors of 0.4% and 0.2% respectively. From these, we can calculate the theoretical experimental error across the dilution series generated from a stock solution of 10^{-4} g·mL^{-1}. A Poisson probability model[13] for the number of wells containing at least one biomarker molecule was developed and applied to the gp120 experimental results in addition to those reported by de la Rica and Stevens on detection of p24.[4] Taking into account the molecular weight of gp120 being five times that of p24, these results are in agreement with the previous experiment by de la Rica and Stevens.[4] As shown in figure 3, the data reported for gp120 in terms of number of molecules per well reflect the high sensitivity of the assay. Indeed few molecules (nominally c.a. 5) were sufficient to generate a detectable and reproducible colour change at 10^{-17} g·mL^{-1} of gp120. At 10^{-18} g·mL^{-1} gp120, we observe one blue well and two red wells resulting in a large standard deviation. This is

understandable because we approach the limit of single molecule detection and the uncertainty attributable to sampling is large. In fact, this result corresponds to the most likely outcome according to statistical models based on the Poisson distribution (Table 1; see also ESI). A statistical analysis shows that in the present two cases the most likely outcomes are robust to estimated random and systematic errors of measurement; this was not self-evident in view of the error propagation through the series of dilutions

It is very important to point out that the present protocol results in a binary outcome, i.e. in the presence of a very low amount of antigen (approaching the single molecule limit), nanoparticle growth leads to a blue solution or conversely not enough molecules are present and the solution appears red. The assay does not show any notable differences in optical properties above the LOD. As pointed out above, the success of the protocols likely depends on several factors such mixing time, equipment precision, and temperature amongst others. Future work will be devoted to isolating these parameters and to understand their impact on the final outcome, with the aim of elucidating a fuller understanding of the exact mechanism.

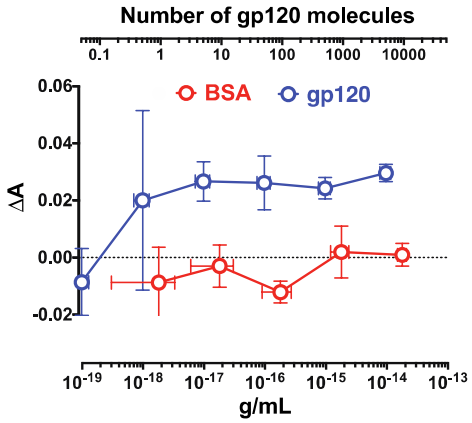

Figure 3. **Decrease in absorbance measured at 550 nm with respect to the blank for samples containing gp120 at different concentrations.** Note the second x-axis reporting the number of gp120 molecules can only be applied for the gp120 data, BSA data should be corrected by the mass ratio between the two proteins. Y error bars are calculated as SD over 3 independent experiments. The X error bars are calculated from the pipette tolerance as supplied from the manufacturer and propagated across the dilution series but do not include variability attributable to sampling.

Table 1: Poisson-binomial model applied to plasmonic ELISA to calculate the probability of a given number of wells containing at least one biomarker molecule. Probabilities calculated using the Poisson-Binomial model and based on nominal concentrations at the 10^{-18} g·mL^{-1} dilution of 2.5 per well for p24 (as in de la Rica and Stevens[4]) and 0.5 per well for gp120. Probabilities of the most likely outcome corresponding to results observed in here for gp120 and in de la Rica and Stevens[4] for p24 are highlighted in bold, and in both cases this is precisely the outcome observed (refer to ESI for further details). These calculations suggest that the results observed in the two experiments are consistent.

Biomarker	Wells containing biomarker	Dilution (g·mL^{-1})		
		10^{-17}	10^{-18}	10^{-19}
gp120	0	0.000	0.223	-
	1	0.000	**0.434**	-
	2	0.020	0.282	-
	3	**0.980**	0.061	-
p24[1]	0	0.000	0.001	**0.472**
	1	0.000	0.019	0.402
	2	0.000	0.207	0.114
	3	**1.000**	**0.773**	0.011

[1]As reported by de la Rica and Stevens[4] and included for comparative purposes.

Conclusions

In conclusion, we have demonstrated that plasmonic ELISA can detect gp120 at ultralow concentrations when an independent laboratory performed the experiments. Furthermore we have corroborated that a change in the concentration of H_2O_2 in the growth solution as small as 0.05 μM can yield nanoparticle solutions of radically different tonality.

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Notes and references

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† All the experiments shown in this paper were exclusively performed by DC without any intervention at all from the other authors. GB directly supervised the experiments. RdlR, MMS and GB designed and interpreted the experiments. RESB and MWF conducted the statistical and error analyses. All authors contributed to the writing of the manuscript.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x/

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