Inverse sensitivity of plasmonic nanosensors at the single-molecule limit

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Abstract

Recent work using plasmonic nanosensors in a clinically relevant detection assay reports extreme sensitivity based upon a mechanism termed *inverse sensitivity*, whereby reduction of substrate concentration increases reaction rate, even at the single-molecule limit. This near-homœopathic mechanism contradicts the law of mass action. The assay involves deposition of silver atoms upon gold nanostars, changing their absorption spectrum. Multiple additional aspects of the assay appear to be incompatible with settled chemical knowledge, in particular the detection of tiny numbers of silver atoms on a background of the classic ‘silver mirror reaction’. Finally, it is estimated here that the reported spectral changes require some 2.5 × 10^{11} times more silver atoms than are likely to be produced. It is suggested that alternative explanations must be sought for the original observations.
Figure 1: **Summary of assay reactions.** The silver atoms generated can either be deposited on gold nanostars (right branch), which leads to a blueshift of the absorbance spectrum, or can aggregate in free solution (‘nucleation’, left branch), in which case they do not affect the solution absorbance.

1 **Introduction**

Rodriguez-Lorenzo et al. [1] report an ultra-sensitive method for detecting analytes that can be recognised by an antibody. The PSA protein is used to demonstrate the technique. The basis of the assay is for the antigen to be recognised by antibodies conjugated with the glucose oxidase enzyme (GOx), which then produces hydrogen peroxide. The H$_2$O$_2$ in turn reduces silver ions, the resulting silver atoms being deposited on gold nanoparticles (‘nanostars’). The deposition is detected by a blueshift of the absorption spectrum of the solution of gold nanoparticles. The reactions are summarised in Fig. 1 of this analysis.
2 Inverse sensitivity

Rodriguez-Lorenzo et al. [1] report bizarre, less-is-more reaction kinetics, according to which the reaction proceeds more quickly as the substrate concentration is reduced close to zero. In their own words (from the abstract of their paper):

However, because conventional transducers generate a signal that is directly proportional to the concentration of the target molecule, ultralow concentrations of the molecule result in variations in the physical properties of the sensor that are tiny, and therefore difficult to detect with confidence. Here we present a signal-generation mechanism that redefines the limit of detection of nanoparticle sensors by inducing a signal that is larger when the target molecule is less concentrated.

The approximate form of the kinetics is sketched in Fig. 2A. As the substrate concentration is increased, the reaction rate rises abruptly from zero and then declines logarithmically (the authors’ plots are semi-logarithmic) from a peak at extraordinarily low concentrations. In the GOx-detection experiment of their Fig. 1c, that peak occurs at a concentration where less than one molecule of GOx is expected to be present in the reaction volume (this is calculated in the next section). In contrast, the law of mass action states that the reaction rate is proportional to the product of the substrate concentrations (more accurately activities). Since only the analyte concentration is varied in the present experiments and it only appears with first-order kinetics, the reaction rate should simply be proportional to the analyte concentration at low concentrations. ‘Inverse sensitivity’ appears to be spectacularly incompatible with the law of mass action.

The ‘explanation’ offered by Rodriguez-Lorenzo et al. [1] for this discrepancy is that spontaneous nucleation of pure silver nanoparticles at high concentrations bypasses the deposition of silver on the gold nanostars. Inspection of the assay reactions (Fig. 1 of this analysis) shows that only the right-hand branch, in which silver ions are deposited on the gold nanostars, leads to the blueshift used to detect the analyte. Thus, the presence of a competing nucleation reaction can only reduce reaction sensitivity (Fig. 2B

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1There is maybe some uncertainty regarding the dependence of the blueshift of the absorbance peak on the amount of silver deposited, but the former can be assumed to be an increasing function of the latter, so the conclusions reached here would be unaffected by the details of the relation.
Figure 2: **Inverse sensitivity.**  
**A.** The approximate form of reaction kinetics reported by Rodriguez-Lorenzo et al. [1] is depicted by the magenta curve (**Inverse sensitivity**); the plot axes are linear. The reaction rate increases sharply at a very low threshold and then decays logarithmically with substrate concentration. In some experiments in the paper, the peak rate occurs at dilutions where *less than one analyte molecule* is expected to be present. In contrast, according to the law of mass action, the rate should be proportional to the product of the substrate concentrations (or activities).  
**B.** Whatever the substrate-dependence of silver production, the presence of a *nucleation* reaction that consumes silver atoms in competition with the detected *deposition* reaction can only *reduce* the overall sensitivity of the reaction.  

of this analysis), irrespective of the dependence of silver production, nucleation and deposition on analyte concentration. Nucleation cannot increase assay sensitivity\(^2\).

\(^2\)It is possible that in their conceptual argument the authors have confused the *slope* of the analyte-blueshift curve, which could conceivably become negative at high silver concentrations, with the *absolute* blueshift. In any case, what matters is the sensitivity at *low* analyte concentrations, where the nucleation reaction is unlikely to proceed.
3 Sensitivity and noise

The assay is reported to have extraordinary sensitivity and exceptionally low noise levels.

Fig. 2c of the paper (note that this and all figure references below are to those in ref [1]) reports the detection of the difference between zero glucose oxidase and $1 \times 10^{-20}$ g/ml glucose oxidase, which represents an average of 0.04 molecules of GOx (MW = 160 kDa) per ml. The precise reaction volume is not reported in the paper, but would need to have been about 10 ml to have had a 50% chance of containing a single molecule of GOx. A reaction volume of 1 ml was used elsewhere in the paper. As no statistics are given for this figure, this observation may have been a lucky event whose replication was never attempted.

Fig. 4 shows a quantification of the variability of the assay. In both panels a and b, we see that $1 \times 10^{-18}$ g PSA in the reaction volume of 1 ml is detected with fantastic precision compared to the amount of $1 \times 10^{-19}$ g. At both concentrations, the standard deviation of the assay signal is in most cases smaller than the symbol and in all cases smaller than a few percent of the maximum signal. But $1 \times 10^{-18}$ g/ml of PSA represents an average of just 23 molecules in the reaction volume of 1 ml. Such small quantities would necessarily exhibit stochastic variation in the number of molecules present. By Poisson statistics, 23 molecules should be associated with a standard deviation of $\sqrt{23}$, equivalent to 21%. This Moreover represents a minimum. The signal amplification required to detect such small quantities would certainly contribute additional (high levels of) noise. Yet the authors consistently report improbably low standard deviations.

This amazing sensitivity is at odds with a publication that predated Rodriguez-Lorenzo et al. Li et al. [2], who used a variation of the present assay to detect glucose (of which more below) with an excess of GOx (as opposed to detecting GOx with excess glucose). Li et al. report a detection threshold of 10 nM (although their Fig. 1 suggests that values in the micromolar range might be more realistic). Even if a GOx molecule will obviously produce more silver than a glucose molecule (estimated below), the difference between the claimed detection thresholds for Li et al. and Rodriguez-Lorenzo et al. is extreme: $1 \times 10^{-8}$ M vs. $6 \times 10^{-23}$ M, a factor of $6 \times 10^{14}$.

That single-molecule sensitivity is rendered even more unexpected by another result in Li et al. [2]. In their Fig. S7, they compare the abilities of glucose and $\text{H}_2\text{O}_2$ to reduce silver ions. They report that $\text{H}_2\text{O}_2$ is much less effective. From this we deduce that each molecule of $\text{H}_2\text{O}_2$ is by no means
guaranteed to reduce a silver ion. A poor yield at this stage of the assay would reduce its sensitivity even further, making single-molecule detection even more implausible.

4 Silver mirror reaction

Another problem is that the deposition of silver is triggered using a mixture of AgNO$_3$ and NH$_3$. The authors describe silver being deposited on the gold nanoparticles (or aggregating via nucleation and growth) as a result of reduction by the H$_2$O$_2$ produced by glucose oxidase. In order for this to allow detection of single molecules, a strict requirement is that absolutely no silver at all be deposited in the absence of GOx and the H$_2$O$_2$ it produces. However, it turns out that the assay reaction probably contained two sources of reductants that were neither acknowledged nor apparent in the results. Either of these sources would generate background reductant concentration in excess of that arising during the claimed detection of single analyte molecules.

The authors seem to have been unaware that they were using a classic classroom reaction called the ‘silver mirror reaction’. The mixture of AgNO$_3$ and NH$_3$ is called Tollen’s reagent and is used to detect aldehydes, whose presence triggers the deposition of a visually impressive silver layer on any available surface. A nice description of the reaction for motivating secondary school chemistry classes can be found on the Royal Chemistry Society website [3]. As demonstrated in that example, the reaction will produce a positive in the presence of glucose, which has an aldehyde form in solution. The problem is that in the assay of Rodriguez-Lorenzo et al., 100 mM glucose is present as the substrate for glucose oxidase. It seems inconceivable that it would not produce much more silver deposition than the tiny amounts of H$_2$O$_2$ produced by a few glucose oxidase molecules.

The paper by Li et al. [2] provides direct support for our assertion that glucose would reduce silver and generate a signal, because they apply this assay precisely for the detection of glucose! The 100 mM glucose present in all experiments of Rodriguez-Lorenzo et al. would therefore generate a saturating reduction of silver, against which background it would presumably be impossible to detect single-molecule signals. In any case, these expected and demonstrated background signals are simply absent from the results reported by Rodriguez-Lorenzo et al.

The assay potentially contains a second source of reductant able to swamp single-molecule signals. Luo et al. [4] report that gold nanoparticles
can catalyse the oxidation of glucose, producing $\text{H}_2\text{O}_2$. This catalysis is quite efficient for bare nanoparticles. Some coatings of the gold can prevent the catalysis and this may pertain in the experiments of Rodriguez-Lorenzo et al. However, the covering would have to be perfect to allow single-molecule detection.

5 Nanoparticle numbers

There are two further issues with quantitative aspects of the assay as reported by the authors. I give a brief overview before expounding the detailed arguments.

The first problem is that the quantities of enzyme involved will produce absolutely tiny amounts of $\text{H}_2\text{O}_2$ and correspondingly tiny amounts of silver—enough to deposit only a single atom on each of a very small fraction of the gold nanoparticles present. It is extremely unlikely that addition of a single atom will detectably change the absorbance spectrum of the nanoparticle.

The second and related problem is that the expected large fraction of unmodified nanoparticles appears not to contribute to the reported spectrum. Because the assay signal is the absorbance of a dilute solution of nanoparticles, each nanoparticle will contribute approximately independently to that absorbance. In the absence of silver deposition, a control spectrum is obtained. Modified nanoparticles would have a different spectrum depending on the degree of modification. If a solution contains modified and unmodified nanoparticles, a simple mixture of the two spectra should be obtained. However, even under conditions where a very large fraction of nanoparticles must have been unmodified, their dominant contribution to the mixture spectrum was apparently absent.

The more detailed explanations follow below and in the next section.

The assay is in two stages. $\text{H}_2\text{O}_2$ is produced by the action of GOx attached to the nanostars for 1 hour, then the silver ions are added to trigger the silver deposition and/or nucleation, which are allowed to proceed for another 2 hours. The precise reaction mixture for the second stage is 0.1 mM $\text{AgNO}_3 + 40$ mM $\text{NH}_3$ added to the 10 mM MES buffer (pH 5.9) already present.

A first remark is that GOx is presumably totally inactivated by the basic pH $\geq 10$ of the second stage after addition of $\text{NH}_3$ (see Fig. 5 of ref [5]). It also seems that GOx is strongly inhibited by silver ions [6]. So it is unnecessary to consider $\text{H}_2\text{O}_2$ and silver produced next to the nanostars, just
the H$_2$O$_2$ concentration existing in the bulk solution at the end of the first stage and the silver it produces during the second stage. There is therefore no kinetic advantage in attaching the GOx to the nanostars.

What is the concentration of H$_2$O$_2$? The authors have omitted details about the GOx used, so we’ll assume it is the most active one available from Sigma: G7141, with an activity of 100000–250000 units/g [7]. The unit definition is:

One unit will oxidize 1.0μmole of β-D-glucose to D-gluconolactone and H$_2$O$_2$ per min at pH 5.1 at 35 °C equivalent to an O$_2$ uptake of 22.4μl/min. If the reaction mixture is saturated with oxygen, the activity may increase by up to 100%.

Another Sigma page [8] indicates that the final glucose concentration under the conditions for the unit definition is 1.61%w/v or 90 mM—similar to the 100 mM used by the authors.

Consider Fig. 2 and in particular the spectra in panel b for zero glucose oxidase (black, blue) and $1 \times 10^{-20}$ g/ml GOx (red). Using the enzyme activity values just given, it can be calculated that this low concentration of GOx would produce an H$_2$O$_2$ concentration of $1.5 \times 10^{-16}$ M after 1 hour. Generously assuming the production of one silver atom per H$_2$O$_2$ molecule, $9 \times 10^4$/ml silver atoms would be produced. (Above, we mentioned results that suggest that this conversion is far from complete, which would result in many fewer silver atoms.)

We now calculate the number of nanostars. The concentration of nanostars is presumably the same as in the assays: [Au] = 0.25 mM (Methods). We’ll also need the following values: nanostar diameter 60 nm (Fig. 2a; Methods), so radius 30 nm; density of gold 19.3 g/ml; atomic weight of gold 197. The volume of a nanostar (assumed spherical) would be $1 \times 10^{-16}$ ml. This would contain $2 \times 10^{-15}$ g of gold or $1 \times 10^{-17}$ moles. So 1 ml of 0.25 mM [Au] should contain $2.3 \times 10^{10}$ nanostars.

There would therefore only be enough silver to deposit just one atom on each of 0.0004% (about 1 in 260000) of the nanostars. The rest would have no deposited silver. As mentioned above, such a minimal modification as deposition of a single silver atom is very unlikely to produce a detectable change of absorbance of a nanostar; we estimate in the next section the amount of silver deposition necessary to create the spectral changes reported.

Furthermore, at least 99.9996% of the nanostars must be unaltered. They would necessarily have the same spectrum as those in the zero GOx control. The small admixture of the 0.0004% nanostars each modified by
a single silver atom will presumably make very little difference. Yet hugely
different spectra are reported. Please compare again the black and red
spectra, and consider that the difference is supposed to result from 0.0004% of
nanostars having a single silver ion deposited on them. In reality, a
spectrum dominated by the majority unmodified nanostars and therefore
almost identical to the control spectrum would be expected.

A similar, if slightly less extreme, problem exists for the PSA assays of
Fig. 4, which show very strong signal at $1 \times 10^{-18}$ g/ml PSA and for which
the exact gold concentration is specified (i.e. $[Au] = 0.25$ mM). If we make
the very generous assumption that each PSA molecule has attached to it
100 GOx molecules, still only about 1 in 4 nanostars will receive a solitary
silver ion, with the rest being unaltered.

### 6 Expected blueshift

I now estimate the amount of silver deposition required to produce the
reported spectral shifts of nanostar absorbance.

In general, unadorned gold nanoparticles are associated with a (rela-
tively) red absorbance peak, while those with silver shells display a peak
that is closer to the blue. The spectral peaks in Rodriguez-Lorenzo et al. are
rather red-shifted compared to most of the spectra in the literature; presum-
ably because of the relatively large size of the present nanoparticles.

The key observation is that under conditions where silver is supposed to
have been deposited on the nanostars, there is no sign of the spectral peak
attributable to the unmodified gold nanostars. In particular, the spectrum
for $1 \times 10^{-20}$ g/ml GOx of Fig. 2b (red) shows no sign of the peak seen
in the control spectra (black and blue). This suggests that the majority
of nanostars have been coated with a silver layer sufficient to obscure the
gold peak. I’ll try to estimate this thickness with reference to work in the
literature.

This simple calculation will assume spherical nanoparticles. Conve-
niently, the densities and atomic weights of silver (10.3 g/l and 108) are
such that metallic gold (19.3 g/l and 197) and silver contain very similar
numbers of atoms per unit volume.

Kim et al. [9] measure spectra before and after silver deposition. They
report the spectra of gold-core nanoparticles with silver shells for different
mole fractions of the two metals. By a little elementary geometry, we can
obtain the thickness $T_{Ag}$ of the silver shell from the radius of the gold core
($r_{Au}$) and the silver mole fraction ($m_{Ag}$):
\[ T_{Ag} = r_{Au} \left( \frac{3}{2} \sqrt[3]{1 + m_{Ag}(1 - m_{Ag})} - 1 \right). \]

The volume of silver per nanoparticle is

\[ V_{Ag} = \frac{4}{3} \pi ((r_{Au} + T_{Ag})^3 - r_{Au}^3) \]

and if the volume is in cubic metres, the number of silver atoms is then

\[ N_{Ag} = \frac{1 \times 10^7 \times 10.3V_{Ag}}{6 \times 10^{23} \times 10^8}. \]

Fig. 2 of ref [9] shows the growth of a blueshifted peak that eventually obscures the red peak from the gold core. Two particle sizes of diameters 13 nm and 25 nm were tested. With the smaller one, none of the silver mole fractions tested obscured the gold peak in the way seen in Fig. 2b of Rodriguez-Lorenzo et al. Such an effect is, however, observed with the larger particles. The largest silver mole fraction for which the gold peak is still larger than the silver one (and therefore still definitely detectable) is 0.25. This corresponds to an average silver layer thickness of about 1.3 nm. Even on such small nanoparticles this would imply \(1 \times 10^5\) silver atoms per nanoparticle. (Note that the nanostars are larger and have an increased surface area because of their shape, but my aim here is to avoid overestimating the number of silver atoms.)

If there are \(2.3 \times 10^{10}/\text{ml}\) nanostars (see previous section), that would imply that 1 ml of solution would require deposition of at least \(2.3 \times 10^{15}\) silver atoms to achieve the observed spectral shift. The discrepancy with the maximum number of \(9 \times 10^4\) that could be produced by \(1 \times 10^{-20}\) g/ml GOx (calculated above) is a mere factor of \(2.5 \times 10^{11}\). Beside this large number, the various imprecisions in my calculation (size of the nanostars, any specific plasmonic effects associated with the vertices of the nanostars) are probably irrelevant.

7 Summary

The premise of inverse sensitivity in Rodriguez-Lorenzo et al. [1], that a competing reaction can increase the sensitivity of an assay at the single-molecule limit, seems to be kinetic nonsense. They report detection of GOx when the reaction volume would only rarely have contained a single molecule. The detection of small numbers of analyte molecules does not display the stochastic
variability expected. The detection of tiny numbers of silver atoms is implicitly claimed, but the assay conditions contain a textbook reaction for producing silver atoms in large quantities independently of the analyte detection mechanism. The complete disappearance of the spectral peak of gold nanostars unmodified by silver atoms is hard to reconcile with the estimate that only a tiny fraction of stars will receive even a single silver atom. The apparent discrepancy between the amount of silver likely to be produced by analyte detection and that estimated to be required to produce the changes of the absorbance spectrum is a factor of at least $2.5 \times 10^{11}$. The authors should provide a more plausible explanation for their observations.

8 Acknowledgements

This analysis is based upon comments I posted on the PubPeer platform as Peer 2: https://pubpeer.com/publications/3E8208F0654769A44C22D4E78DA2B8. My attention was drawn to the article by the initial comments on the paper by Peer 1.
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