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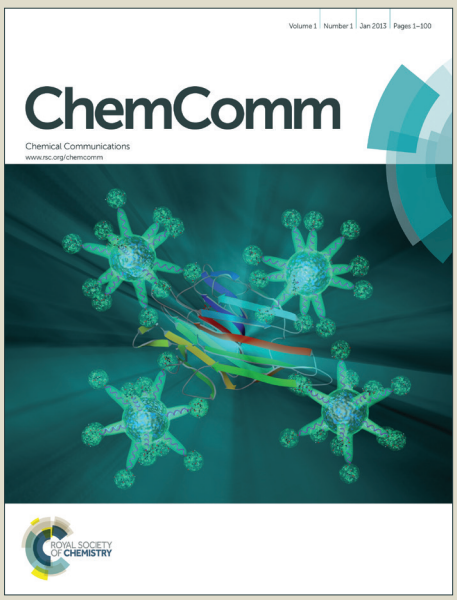





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# Aptamer-mediated 'turn-off/turn-on' nanozyme activity of gold nanoparticles for kanamycin detection

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**A new ultrafast and highly sensitive 'turn-off/turn-on' biosensing approach that combines the intrinsic peroxidase-like activity of gold nanoparticles (GNPs) with high affinity and specificity of a ssDNA aptamer is presented for efficient detection of a model small molecule kanamycin.**

With the advent of synthetic and chemical biology, non-stereotypical role of nucleic acids recently came into existence in the form of outstanding molecular recognition elements (MREs), typically known as aptamers.[1] Aptamers are single stranded DNA or RNA molecules that are generated through an *in-vitro* selection process called **S**ystemic **E**volution of **L**igands by **EX**ponential enrichment (SELEX).[1] These molecular ligands have the ability to recognise their cognate targets with high affinity and specificity. This property makes aptamers an ideal tool to detect a variety of targets including but not limited to small molecules such as antibiotics, pesticides, amino acids and nucleotides; large biomacromolecules such as proteins and nucleic acids; as well as whole cells.[2]

Due to the outstanding target recognition ability of aptamers, it is not surprising that aptamers bound onto the surface of nanomaterials are now regularly employed for development of a range of biosensing platforms.[1a-c,2a,3] Gold nanoparticles (GNPs) offer an edge over other nanomaterials to develop colorimetric sensing platforms, particularly due to their well-established synthesis and surface modification protocols as well as their remarkable surface plasmon resonance (SPR) properties that allow a visual output of the sensing events when GNPs change their colour in the presence of an analyte due to nanoparticle agglomeration.[4] However, one of the major limitations associated with this GNP-based colorimetric detection is the tendency of GNPs to non-specifically aggregate in the presence of salt and other molecules present in the complex biological fluids. To some extent, this limitation has been overcome by employing aptamer-coated salt-resistant GNPs, which did not aggregate by salt in the absence of aptamer target.[2a,3b] Interestingly, in this case, in the presence of the cognate target, the aptamer could leave the surface of GNPs to bind to its target, which allowed salt-induced controlled aggregation of GNPs to be used as a strength of this biosensing platform. Overall, although several biosensing platforms employ size-dependent change in SPR of GNPs, the non-specific aggregation of GNPs remains a challenge.

More recently, GNPs were discovered for their ability to oxidise the peroxidase substrates in the presence of $H_2O_2$ to form coloured reaction products.[5] This intrinsic peroxidase-like activity of GNPs is typically independent of gold aggregation behaviour which makes it one step closer to traditional biochemical assays such as ELISA. While this new enzyme-mimicking activity of GNPs has spurred a major interest to develop biosensing platforms, these nanozyme biosensors have been predominantly restricted for non-specific detection of glucose and $H_2O_2$.[5d,6] This non-specificity is most likely due to the lack of a molecular recognition element in previous reports.

Since the peroxidase-like activity of GNPs results from interaction of GNP surface with the peroxidase substrate, the hypothesis behind the current work is that the peroxidase-like activity of a GNP can be turned-off by blocking its surface through the adsorption of ssDNA aptamer molecules, thereby providing a shielding effect against interaction of GNP with the peroxidase substrate. However, in the presence of a suitable target analyte, this peroxidase-like activity can again be turned-on, as the high affinity of the aptamer to the target will induce aptamer desorption from the GNP surface to allow aptamer-target binding event, subsequently permitting the GNP to resume its peroxidase-like activity. In the current study, we utilise this phenomenon to demonstrate proof-of-concept detection of small molecules by developing a simple, rapid, sensitive and highly specific colorimetric assay for detection of kanamycin. Kanamycin is an aminoglycoside antibiotic that is widely used in veterinary medicine.[7] Considering the potential

transfer of kanamycin to humans in the food chain and its serious side effects, kanamycin contamination in food is monitored in most countries.[7,8] For instance, the maximum residue limit established by the European Commission for kanamycin in milk is 150 μg/kg, while in meat it ranges from 100-2500 μg/kg (muscle and fat: 100; liver: 600; kidney: 2500 μg/kg).[9] This provides a strong rationale to develop new biosensors for detection of small molecules such as kanamycin.

To develop kanamycin-specific biosensor, the current study employed tyrosine-reduced GNPs as a peroxidase nanozyme[10] and Ky2 aptamer as a kanamycin-specific MRE (ESI, **Fig. S1**).[3b] Schematic in **Fig. 1** illustrates the principle of the aptamer and nanozyme-based colorimetric biosensing assay, wherein the pristine GNP shows intrinsic peroxidase activity, resulting in oxidation of the colourless substrate 3,3,5,5-tetramethylbenzidine (TMB) to a purplish-blue product (step A). As shown in step B, the blocking of GNP surface through conjugation of ssDNA Ky2 aptamer leads to the inhibition of this peroxidase activity, which remains 'turned-off' in the absence of its cognate target, kanamycin, thereby resulting in no colour change. Conversely, in the presence of kanamycin, the high affinity and specificity of Ky2 aptamer towards kanamycin leads to desorption of the aptamer molecules from the surface of GNP, subsequently again 'turning-on' the nanozyme activity of GNP that catalyses oxidation of TMB to a coloured product (step C). This new biosensing strategy therefore represents a simple, one step and straightforward approach for detection of small molecules.

The physico-chemical characterisation of pristine GNP, aptamer-conjugated GNPs (GNP-Ky2) and aptamer-conjugated GNPs on exposure to kanamycin (GNP-Ky2+Kan) is shown in supporting information (ESI, **Fig. S2**). The UV-visible absorbance spectroscopy, dynamic light scattering and TEM measurements confirm that GNPs remain stable during different sensing steps. Further, the biosensor optimisation data to assess the appropriate sensing temperature as well as GNP and aptamer concentrations are shown in ESI (**Fig. S3 and S4**).

**Fig. 2** compares the peroxidase-like activity of optimised GNP-Ky2 nanoconjugate in the presence of specific and non-specific target analytes. During this assay, when pristine GNPs were exposed to a mixture of TMB and $H_2O_2$, it resulted in a visual colour change within 8 min due to the oxidation of TMB. This visual readout can also be quantitatively monitored by measuring the product absorbance at 650 nm. In comparison with pristine GNP (normalised as 100% activity), the activity of GNP-Ky2 (test reagent) significantly reduces to 9.84% of the original activity. When GNP-Ky2 is introduced to a solution containing aptamer target kanamycin, the nanozyme activity gets resumed, leading to a 73.86% activity, which is 7.5 times higher than that of the test reagent (GNP-Ky2) itself. To check the specificity of the test reagent, GNP-Ky2 is introduced to other non-specific small molecules such as penicillin, ampicillin and streptomycin (structures shown in ESI, **Fig. S5**). This showed only marginal change in activity in the presence of these non-specific analytes [penicillin (13.54%), ampicillin (15.15%) and streptomycin (18.55%)]. Notably, streptomycin and kanamycin belong to the same aminoglycoside group of antibiotics which have high structural resemblance. Therefore, it is not surprising that among different non-specific analytes, the non-specific activity, albeit minor, was found highest against streptomycin. The above spectroscopic observations also correlate well with the visual readouts of the sensing event. Optical images in **Fig. 2** show that in the absence of cognate target or in the presence of non-specific antibiotics, the ruby-red colour of aptamer-functionalised GNPs is retained for at least up to 30 min. This cross-reactivity study provides convincing evidence on the ability of the Ky2 aptamer to detect kanamycin with high specificity by proposed approach.

A major difference between the proposed biosensing approach and the previous approaches that employed aptamer technology with GNPs is that our platform provides a colorimetric read-out of an oxidised peroxidase substrate whereas other approaches are dependent on SPR change in GNPs due to salt-induced aggregation of GNPs.[3a-c,5d,11] Since efficient salt-induced aggregation of GNPs will require almost complete removal of aptamers from the GNP surface, previous strategies have been limited in sensitivity as complete aptamer removal warrants high concentrations of the target analyte.[3b,c,12] Additionally, the long incubation time (typically 1-2 h) required during salt-induced aggregation also limits the effectiveness of previous strategies.[3b,c,12]

In the current study, the use of the inherent nanozyme activity of GNPs for biosensing eliminates the need for salt-induced aggregation leading to improved sensitivity and faster readouts. The sensitivity of kanamycin detection using nanozyme-aptamer strategy is evident from **Fig. 3**, which shows that GNP-Ky2 system has the potential to detect as low as 1 nM kanamycin. Further calculations revealed an outstanding performance of proposed nanozyme biosensor in context of limit of detection (LoD), limit of quantification (LoQ), linearity, dynamic range, precision and accuracy (ESI, **Fig. S6**). The GNP-Ky2 nanozyme biosensor showed an impressive LoD of 1.49 nM, LoQ of 4.52 nM while achieving a high dynamic range within the linear operational limit of 1-100 nM. Further, when tested for 20 repeat events at 5 nM kanamycin concentration, the biosensor was found to operate with a precision of 95.7%, while showing 90% accuracy at 5% confidence level and 95% accuracy at 10% confidence level.

The time taken for detection of analyte molecules is another important parameter for efficient diagnosis. Therefore, real-time evaluation of nanozyme activity towards TMB oxidation in the presence of different analytes was also performed (**Fig. 4**). The GNP-Ky2 test reagent shows much faster rate of activity in the presence of kanamycin compared to that in the presence of other non-specific analytes. In particular, the impressive specificity of Ky2 aptamer towards kanamycin is evident from low activity profile in the presence of streptomycin, which otherwise has high structural similarity to kanamycin. This demonstrates high specificity of this assay while indicating that through appropriate optimisation, it might be feasible to detect small molecules as fast as within 3 min by employing this facile 'turn-off/turn-on' aptamer-controlled nanozyme strategy. Interestingly, at early time points although GNP-Ky2 in the presence of kanamycin shows similar activity as that of pristine GNP, at the later stages of the reaction, GNP outperforms GNP-Ky2. This suggests that at 100 nM kanamycin concentration, some of the Ky2 aptamer molecules remain attached to the GNP surface. Therefore, by increasing the reaction time, concentrations higher than 100 nM of kanamycin may also be potentially detected, which is likely to allow this biosensor to be operational in an even broader dynamic concentration range.

Further, the strength of Ky2-kanamycin interaction during this nanozyme-mediated 'turn-off/turn-on' assay was determined by calculating the apparent dissociation constant (Kd) of this interaction (ESI, **Fig. S7**). Interestingly, the nanozyme activity of GNP-Ky2 in the presence of increasing concentrations of kanamycin revealed a typical Michaelis-



 

Menten behaviour that is commonly observed for biological enzymes.[Ref] While this behaviour suggests that the nanozyme activity of GNPs mimics true biological enzymes, linear regression analysis of this interaction in our assay showed a Kd value of 8.38 nM, which is seven times lower than that previously reported for other assays,[3b] thereby suggesting slower off-rate and extremely high affinity of Ky2 for kanamycin in the proposed assay. Furthermore, to ascertain that this high affinity of Ky2 aptamer towards kanamycin is governed by its specific nucleotide sequence, we created three variants of original Ky2 aptamer by mutating the loop as well as the stem regions of the aptamer (ESI, **Fig. S1 and S8**). When these mutated aptamers-modified GNPs were employed for kanamycin sensing, none of them could 'turn-on' the nanozyme activity of GNPs in the presence of kanamycin. This suggests that if any component of Ky2 sequence is mutated, the aptamer loses its ability to recognise kanamycin, implying that both the loop and the stem regions of Ky2 aptamer are critical to retain its high affinity towards kanamycin.

In summary, we have demonstrated a new 'turn-off/turn-on' biosensing strategy by exploiting the intrinsic peroxidase-like activity of GNPs and combining this activity with high affinity and specificity of ssDNA Ky2 aptamer towards a small molecule, kanamycin. This novel assay not only gives a rapid visual readout within 3-8 minutes with high selectivity, it also allows highly sensitive quantitative detection of kanamycin with LoD and LoQ of 1.49 nM and 4.52 nM, respectively, while successfully operating within the linear dynamic range of 1-100 nM. This makes the proposed biosensing platform at least 15 fold more sensitive and 20 times faster than the conventional salt-induced GNP-aptamer approach.[3b,c,9] The current platform may offer a generic approach for the detection of a range of biomedical and environment-relevant small molecules by employing target-specific aptamers, antibodies or other MREs. This approach may also be extended to other nanomaterials that show intrinsic peroxidase-like or other nanozyme activities.[13]

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† Electronic Supplementary Information (ESI) available: Experimental details, nanomaterial characterisation and chemical structure of aptamer and antibiotics. See DOI: 10.1039/c000000x/

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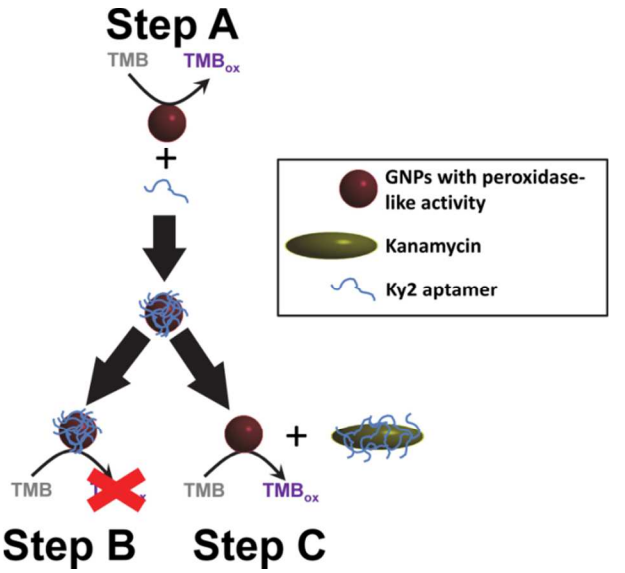


Fig. 1 Schematic representation of the 'turn-off/turn-on' nanozyme activity of aptamer-functionalised GNP for detection of kanamycin. (Step A) shows intrinsic peroxidase-like activity of pristine GNP that gets 'turned-off' after functionalization with Ky2 aptamer and remains 'turned-off' in the absence of kanamycin (Step B), however it gets 'turned-on' again in the presence of kanamycin (Step C).

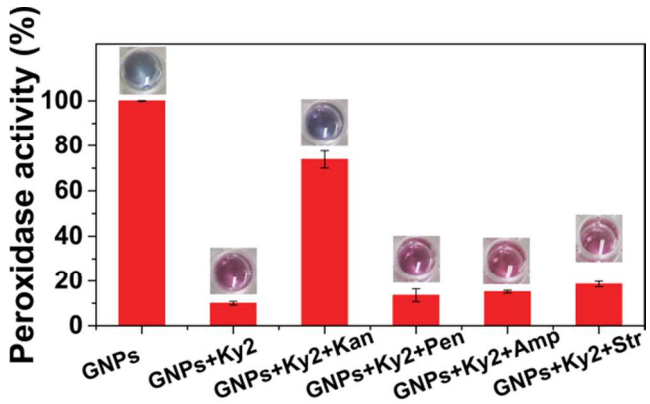


Fig. 2 Peroxidase-like activity of pristine GNP and GNP-Ky2 in the presence of 100 nM target and non-specific analytes after 8 min of reaction. % activity is calculated from $A_{650\ nm}$ of the oxidized TMB while considering the activity of pristine GNPs as 100%. The insets show the optical images of respective solutions after 8 min of reaction. Kan, Pen, Amp and Str correspond to kanamycin, penicillin, ampicillin and streptomycin, respectively.

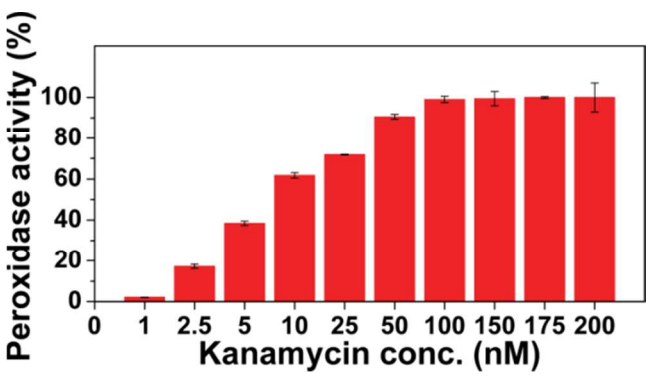


Fig. 3 Peroxidase-like activity of GNP-Ky2 as a function of kanamycin concentration after 8 minutes of reaction. % activity is calculated from $A_{650\ nm}$ of the oxidized TMB after subtracting the background signal of GNP-Ky2 in the absence of kanamycin.

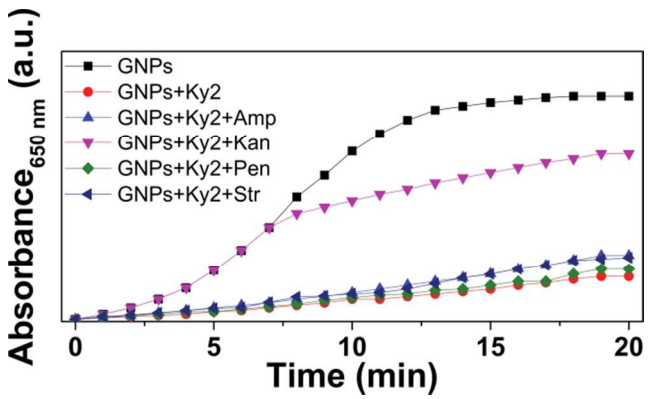


Fig. 4 Time-dependent kinetics showing peroxidase-like activity of pristine GNP and GNP-Ky2 in the presence of 100 nM target and non-specific analytes. Amp, Kan, Pen and Str correspond to ampicillin, kanamycin, penicillin and streptomycin, respectively.