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A glyco-gold nanoparticle based assay for α-2,8-polysialyltransferase from *Neisseria meningitidis*†

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We designed a novel strategy for sensitively detecting the activity of α-2,8-polysialyltransferase (PST) by a combination of ganglioside GD3 functionalized gold nanoparticles and inactive endosialidase. We anticipate that this new method will facilitate the search for PST inhibitors as well as for improved mutant forms of PST in directed evolution experiments.

Polysialic acid (PSA) is a highly hydrophilic and negatively charged biopolymer that is found in bacteria and mammals.[1] In humans, PSA is found predominantly on neural cell adhesion molecules (NCAMs) where it plays a role in modulating cellular interactions during migration and in promoting the plasticity of neuronal cells in early brain development, as well as being involved in cancer metastasis.[2] In bacteria, PSA is a capsular virulence factor and plays a role in the evasion of phagocytosis and in immune clearance by the human host. In addition, since bacterial and human PSAs are identical, PSA has very low immunogenicity and thus has great potential for use in a range of therapeutic applications such as the elongation of circulatory lifetimes of therapeutic proteins.[3]

The enzymes responsible for biosynthesis of PSA are known as polysialyltransferases (PSTs). Both vertebrate and bacterial PSTs[4] are template-independent polymerases, which catalyze the successive addition of sialyl moieties from CMP-sialic acid onto the non-reducing end of a growing PSA chain. While a number of biological studies have been reported for PSTs, the catalytic mechanisms of PSA products are poorly understood, and as yet no 3-D structure of a PST has been determined, in large part due to the difficulties in producing stable and soluble enzymes. The same problems further impede the development of enzymatic PSA synthesis. A key factor limiting the engineering of PSTs is the lack of activity-based, high-throughput screening methodologies for PST activity.

The requirement for an oligosialyl acceptor (primer) and the occurrence of multiple additions render high-throughput assay development for PSTs considerably more challenging than is the case for mono-sialyltransferases (STs).[5] The most widely used PST assays are the radioactivity-based assay using isotopically-labeled CMP-sialic acid as a donor and the CE or HPLC-based assay with fluorescence detection in which a synthetic fluorescent di-sialyllactose (GD3 analogue) or directly derivatized tri-sialic acid is used as an acceptor.[6] While the latter method has provided sensitive and accurate quantification of PST activity, as well as mechanistic insights into PST-catalyzed polymerization, it is instrument-intensive, requires tedious sample pre-treatment, and is not a real-time methodology, thus is not readily adapted to high-throughput screening. Due to the increasing demand for high throughput screens, a simple, sensitive, and homogeneous method for detection of the presence of PSA activity without a need for elaborate washing and pretreatment steps is urgently needed.

Recently, nanoparticles (NPs) have emerged as promising new reagents for biological applications.[7] The unique quantum properties of these nanoscale materials offer excellent prospects for the design of highly sensitive and innovative biosensing devices and machines.[8] Amongst these, gold nanoparticles (AuNPs) have already found use as biosensors,[9] and in nanomedicines,[10] bioseparations,[11] and cell imaging.[12] When used in conjunction with a microtiter plate format, colorimetric assays based on the aggregation of AuNPs represent simple but sensitive methods for the detection of biomolecules and for the evaluation of enzymatic activity.[13] To the best of our knowledge, the use of AuNP-based aggregation to detect enzymatic activity has focused mostly on hydrolytic activity.[13c,d] Herein, we report a novel biosensing strategy to detect bond formation catalyzed by glycosyltransferases, and we exemplify this in an assay for α-2,8-PST activity.

The flow chart of the developed assay is illustrated in Fig. 1. AuNPs decorated with a ganglioside GD3 analog (**1**) were used as a primer for PST-catalyzed polymerization, generating polysialyl structures on the NP surface. Detection is based upon the use of

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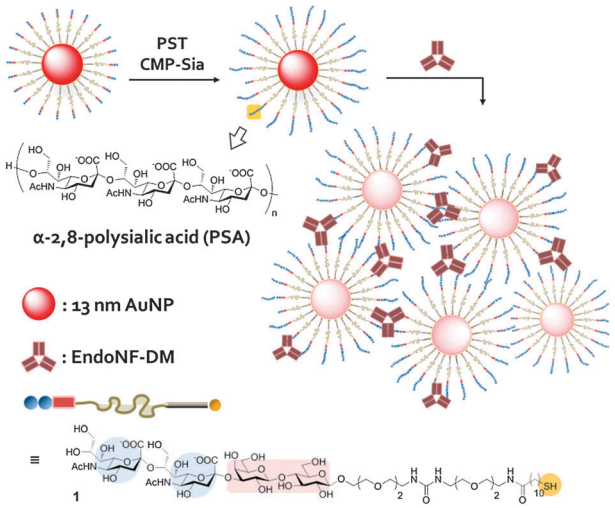

Fig. 1 Schematic representation of the biosensing strategy for α-2,8-PST activity based on EndoNF-DM-mediated assembly of gold nanoparticles decorated with acceptor oligosaccharides that were subjected to enzymatic polysialylation.

an inactive mutant of the polysialic acid specific trimeric phage tailspike endosialidase from the *E. coli* phage K1F, EndoNF-DM, which serves as a cross-linker.[14] Upon addition of EndoNF-DM, the AuNPs with oligo/polymer PSA on the surface undergo aggregation. This triggers a network-like assembly of the PSA-functionalized AuNPs, resulting in aggregation of **PSA@AuNP**s and a significant decrease of intensity in the plasmon resonance absorption peak of the AuNP solution. The simplicity of application of this method offers great potential as a generic approach for activity screening of α-2,8-PSTs and, when other ''cross-linkers'' are used, of glycosynthases and other glycosyltransferases.

To prepare a suitable oligosaccharide ligand to serve as the primer for a PST catalyzed reaction, a long alkane-thiol chain with a hydrophilic oligomeric ethylene glycol linker was chosen as the aglycone not only to facilitate the purification by reverse-phase chromatography but also to establish sufficient steric hindrance to prevent the aggregation of the resulting glyco-AuNPs. Lac$PEG_6C_{10}SH$ **2** was synthesized and then sialylated using α-2,3-ST Cst-I[15] and bifunctional α-2,3/2,8-ST Cst-II,[16] respectively, to afford ganglioside analogs GM3$PEG_6C_{10}SH$ **3** and GD3$PEG_6C_{10}SH$ **1**, as shown in Fig. 2 (for detailed procedures, see ESI†).

The GD3-functionalized AuNPs (**GD3@AuNP**s) were synthesized according to a previously reported procedure[17] by using GD3 ligand **1**. Similarly, **Lac@AuNP** and **GM3@AuNP** were prepared using ligands **2** and **3**, respectively. The UV-visible spectrum of **GD3@AuNP** clearly shows the characteristic plasmon absorption band at 525 nm

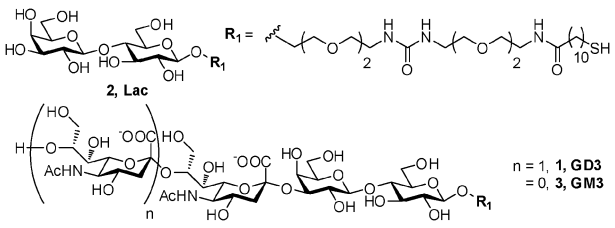

Fig. 2 Oligosaccharide ligands for glyco-AuNPs assembly.

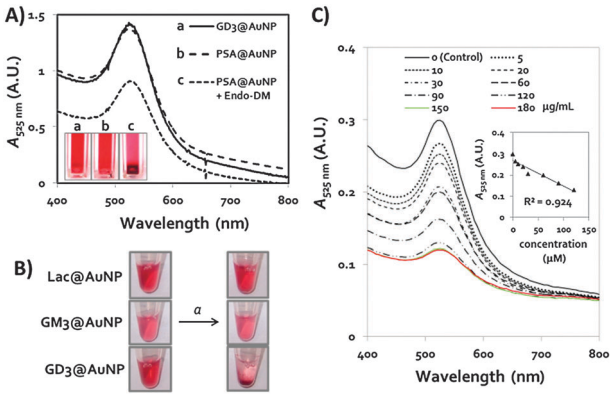

Fig. 3 (A) UV-visible spectra of GD3@AuNP solutions (a) before and (b) after incubation with PST and CMP-sialic acid and (c), following addition of EndoNF-DM. (B) Photographs of the visual detection for the EndoNF-DM-mediated assembly AuNP for PST assay using lactose-, GM3- and GD3-AuNPs as substrates. [a]PST 30 μg mL^{-1}, CMP-sialic acid 1 mM in HEPES 50 mM, pH 7.5, 20 mM $MgCl_2$, 37 °C, 1 h; then EndoNF-DM 3 μM, r.t, 30 min. (C) The absorption spectra of GD3@AuNP solutions incubated with PST at different concentrations in *E. coli* cell lysate and CMP-sialic acid, followed by addition of a fixed concentration of EndoNF-DM; the inset shows the linear plot of the A_{525nm} *versus* the PST concentration.

(Fig. 3A, line a), with similar absorption bands also observed in the spectra of **Lac@AuNP** and **GM3@AuNP** (Fig. S1, ESI†). The lack of an obvious red-shift (from 519 nm to 525 nm) of the maximum absorption band after oligosaccharide modification also indicated that these glyco-AuNPs do not self-aggregate in aqueous media. The **GD3@AuNP**s are well-dispersed in aqueous solution and are stable for at least one week, even in the high ionic strength of 0.1 M sodium chloride solution (Fig. S1, ESI†). The size of the metal core of these glyco-AuNPs is 13 ± 1.2 nm.

The feasibility of such a AuNP-based assay for directly monitoring PSA formation catalyzed by PST was tested with the *Neisseria meningitidis* α-2,8-PST.[3] Incubation of **GD3@AuNP**s (final concentration of 5 nM based on AuNPs)[18] with 300 nM PST and 1 mM CMP-sialic acid in 50 mM HEPES buffer at pH 7.5, containing 20 mM $MgCl_2$, resulted in polysialylation of the NPs, but no visible change in the solution colour or shift in the absorption peak of AuNP, indicating minimal effects of the enzymatic polysialylation reaction on the stability of the **GD3@AuNP**s (Fig. 3A, line b). Addition of the ''cross-linker'' EndoNF-DM[14,19] at a final concentration of 3 μM resulted in the precipitation of **PSA@AuNP** aggregates from the solution and consequently in visible fading of the solution colour over 30 min, quantified as a decrease in the absorbance. This was observed consistently upon aggregation of AuNPs (Fig. 3A, line c). By contrast, no aggregation was observed when **GD3@AuNP**s that had not been polysialylated were incubated with EndoNF-DM. This lack of binding between di-sialic acid and EndoNF-DM is consistent with reports that the EndoNF-DM binding site accommodates at least five residues.[19,20]

Further control experiments were performed to verify that the EndoNF-DM-triggered assembly of the **GD3@AuNP**s was mediated by active enzymatic polysialylation of PST. In these control experiments, either PST or CMP-sialic acid was omitted from the reaction mixture, and indeed no aggregation or precipitation of nanoparticles causing a loss of the intense color in solution was observed after addition of EndoNF-DM (Fig. S2, ESI†). In a further

control experiment, the ineffective "acceptors", **Lac@AuNP** and **GM3@AuNP**, were employed in parallel reactions. As expected, no significant loss of colour in solution due to aggregate precipitation was seen after addition of EndoNF-DM, indicating that, as expected, GD3 is the minimal acceptor required for polysialylation by α-2,8-PST (Fig. 3B).

It is interesting that the more typical colour change of AuNPs from the initial pinkish-red colour of well-dispersed AuNPs to the purple colour that is normally seen with aggregated AuNPs was not observed. Such a colour change is attributed to the change of the inter-particle distance induced by binding and the change of the dielectric constant around the surrounding medium or the shell of the AuNPs. Since PST catalyzes formation of PSA with 4 to 70 sialic acid residues and the EndoNF-DM binds a minimum of 5 to 8 sialic acid residues, it seems probable that the inter-particle distance of the cross-linked AuNPs is not short enough to induce strong coupling between the adjacent nanoparticles. Instead, a decrease in absorbance of the solution at 525 nm resulted from the precipitation of **PSA@AuNP** aggregates, which was facilitated by centrifugation.

The optimal concentration of EndoNF-DM for particle-bound PSA recognition by EndoNF-DM was examined, as shown in Fig. S3 (ESI†). A linear correlation between **PSA@AuNP** absorbance and EndoNF-DM concentration was found. Optimum conditions for EndoNF-DM were an incubation time of 30 min at room temperature in the presence of >3 μM (210 μg mL^{-1}) EndoNF-DM. The linearity of the response with PST concentration in *E. coli* cell lysate was also investigated. As illustrated in Fig. 3C, the absorbance decreased with an increase in PST concentration resulting in a near-linear correlation between the enzyme concentration and the absorbance (Fig. 3C, inset), up to a PST concentration of 1.5 μM. Thus, detection of PST in the nanomolar to micromolar range can be achieved using this assay. Moreover, since **GD3@AuNP**s are stable in a high ionic strength solution, this assay can be performed in the presence of cell lysate without the concern of self-aggregation, making directed evolution screening a reality.

In conclusion, we have developed a novel strategy for sensitive and specific activity assay of α-2,8-PST based on the assembly of **PSA@AuNP**s into network structures mediated by an engineered inactive endosialidase which specifically recognizes the PSA chain. To the best of our knowledge, this study introduces the first high-throughput AuNP-based colorimetric assay for a bond-forming enzyme. The assay has several advantages over traditional techniques: (i) the use of a label-free glycosyl donor avoids problems of radiation and substrate specificity; (ii) visual detection of enzymatic activity is possible in a one-step, highly specific assay; (iii) the enzyme activity can be detected directly from cell lysates, facilitating future screening of directed evolution libraries, and (iv) the screen can be automated and optimized for one-well, one-colony/mutant high-throughput analysis. We expect that this new methodology will play a pivotal role in the development of inhibitors and improved mutants of PST and will provide inspiration for similar assays of other bond-forming enzymes.

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