Bovine Serum Albumin as A Superior "FP-Tag" for High-Throughput Glycosyltransferase Assay

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ABSTRACT: *O*-GlcNAc transferase (OGT) involves in many cellular processes and selective OGT inhibitors are valuable tools to investigate *O*-GlcNAcylation functions, and could potentially lead to therapeutics. However, high-throughput OGT assays that suitable for large-scale HTS, and can identify inhibitors targeting both acceptor, donor sites and allosteric binding-sites is still lacking. Here, we report the development of a high-throughput "FP-Tag" OGT assay with bovine serum albumin as a low-cost and superior "FP-Tag". With this assay, 2-methyleurotinone was identified as a low μM-range OGT inhibitor. This type of assay with BSA as "FP-Tag" would find more applications with other glycosyltransferases.

The addition of a single *N*-acetylglucosamine (GlcNAc) to serine and threonine residues of proteins, terms as *O*-GlcNAcylation, is an important post-translational modification that modulates cellular processes such as cell signaling, transcription, epigenetic modifications, and stress responses.[1-4] Similar as phosphorylation, the *O*-GlcNAcylation dynamics are controlled by a pair of enzymes: O-GlcNAc transferase (OGT) catalyzes the addition of GlcNAc to the target protein using uridine 5'-diphosphate-*N*-acetylglucosamine (UDP-GlcNAc); while β-D-*N*-acetylglucosaminidase (O-GlcNAcase, OGA) removes the O-GlcNAc modifications (Figure 1).[3] Protein phosphorylation usually involved many pairs of kinases and phosphatases; In contrast, O-GlcNAcylation is regulated only by a single pair of OGT and OGA. It has been shown that more than 4000 protein could be O-GlcNAcylated and dysregulation of protein O-GlcNAcylation is closely associated with many diseases such as Alzheimer's disease, diabetes, and cardiovascular diseases.[5-10] Thus, selective OGT inhibitors are valuable tools to investigate O-GlcNAcylation functions, and could potentially be served as therapeutics to treat diseases.[11-14]

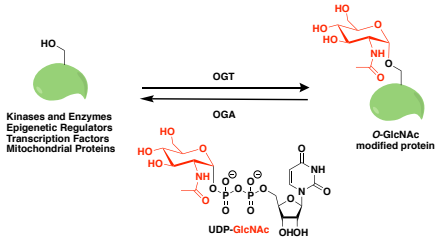

Figure 1. The *O*-GlcNAcylation of proteins regulated by OGT and OGA. OGT catalyzes the addition of GlcNAc to the target proteins such as kinases, epigenetic regulators, transcription factors, and mitochondrial proteins using UDP-GlcNAc as the sugar donor; while OGA removes O-GlcNAc from the modified proteins.

Several high-throughput assays have been developed to screen OGT inhibitors.[15-18] The Walker group developed a high-throughput fluorescence polarization (FP)-based substrate displacement assay for OGT.[15] A high-affinity fluorescent UDP-GlcNAc analog was prepared, and its binding to OGT slows down the tumbling of the fluorophore in solutions. When a plane-polarized light excites the fluorophore, the emitted light would remain at the initial plane because of slow tumbling, resulting in a strong FP signal. During high-throughput screening (HTS), potential inhibitors would compete with the fluorescent UDP-GlcNAc analog for the OGT active site, causing the disassociation between the fluorophore and OGT. The disassociated fluorophore would give a reduced FP signal because of rapid tumbling in solution. This kind of displacement FP assays are high-throughput, not requiring a washing step, thus widely used in HTS campaigns.[19-22]

The disadvantage of such HTS assay is that large amounts of enzyme is needed, and potential inhibitors targeting acceptors or other allosteric binding-sites would likely be missed. In addition, such assays cannot measure inhibition kinetics, and currently, the OGT inhibition kinetics are typically obtained by cumbersome radiolabeled glycosyl donor substrates.[23] The Vocadlo group recently reported a novel, high-throughput, direct fluorescent activity assay for OGT based on streptavidin-coated microplates or streptavidin-coated magnetic beads.[24] However, the assay requires washing steps and those microplates or magnetic beads are expensive for large scale HTS campaigns.

We have recently reported an "FP-Tag" HTS assay for β-Kdo transferase KpsC,[25] inspired by the Paulson group's "catalytic" FP-based sialyl- and fucosyltransferases assay.[26] In such assay, a fluorophore is attached to the acceptor sugar, which freely tumbling in solution to give low FP signal. After KpsC catalyzed the addition of 8-N_3-Kdo to the fluorophore-labeled acceptor sugar, streptavidin-biotin-dibenzocyclooctyne (DBCO) (SBD) was added to covalently attach the fluorophore to Streptavidin by copper-free click reaction. The macromolecule streptavidin, served as an "FP-Tag", restricted the tumbling of the fluorophore, thus giving strong FP reading.

We used this assay to screen against 1000 marine sponge extracts and two μM-range inhibitors were identified. Subsequently, large screens of commercial compound libraries (~145K compounds) were conducted and several potent inhibitors were obtained (unpublished results), demonstrating the robustness of such HTS assay.

This kind of "FP-Tag" HTS assay should be applicable to many other glycosyltransferases. Here we report our effort on developing an "FP-Tag" assay for OGT. We first attached fluorescein to the donor sugar UDP-GlcNAc, and the "FP-Tag" to the acceptor peptide derived from casein kinase II (CKII).[27] (Figure S2A). The advantage of such design is that the reaction progress could be directly monitored by the FP increase. However, we found fluorescein is not well accompanied by OGT, and almost no conversion was observed (Table S1). Therefore, a similar strategy to the KpsC assay was adopted: azide modified donor sugar UDP-GlcNAcN$_3$ and fluorescein-labeled acceptor CKII peptide (FAM-CKII) were employed, and at the end of the reaction, SBD was added to induce the FP response via click reaction (Figure 2A).

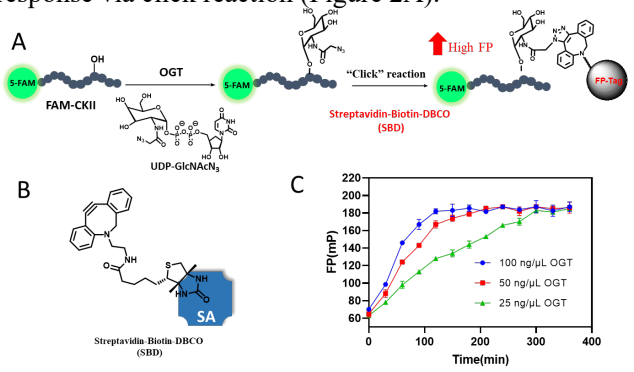

Figure 2. The SBD-based "FP-Tag" assay for OGT. A) GlcNAcN$_3$ is transferred to the fluorescence-tagged acceptor CKII peptide (FAM-CKII) by OGT, and a high FP signal could generate when the SBD "FP-tag" is "clicked" onto the transferred product. B) Structure of the SBD "FP-tag". C) Time course of the OGT reaction progress monitored by the SBD-based "FP-Tag" assay.

The FP signal is the weighted average of the macromolecule-bounded fluorophore and free fluorophore, with bounded fluorophore giving a much strong FP signal. Therefore, to achieve the highest assay sensitivity, as much as fluorophore is needed to attach to macromolecules. With this in mind, we optimized the OGT reaction to contain 1 μM of FAM-CKII and 125 μM of UDP-GlcNAcN$_3$ to fully convert FAM-CKII to FAM-CKII-GlcNAcN$_3$ (Figure S3).

In our KpsC "FP-Tag" assay setup, the SBD solution was directly added to the assay plates at the end of the reactions.[25] However, the addition of SBD solution to the OGT reaction assay plates is not practical because an excess of streptavidin comparing to 125 μM of UDP-GlcNAcN$_3$ is needed to drive the click reaction to completion. This would dramatically increase the screening cost, and also possibly interfere with the FP reading. Therefore, we envision that a small amount of assay solution could be transferred to the SBD solution instead. This transfer would dilute the assay solution, and at the same time introduce the "FP-Tag", SBD, to the recently formed FAM-CKII-GlcNAcN$_3$. We found diluting the assay solution 1000 times (the final total FAM concentration 1 nM) into 2.4 μM of SBD solution gave a robust FP reading of 180 mP after completion of the click reaction.

The progress of the OGT reaction was then monitored by the "FP-Tag" assay. The reaction was started by the addition of OGT, and aliquots of assay solution were diluted into SBD solution containing 1 mM UDP (to stop the OGT reaction) as UDP is a potent OGT inhibitor.[28-29] After the click reaction went to completion, FP signals were measured. As showed in Figure 2C, after 5 h, three different concentrations of OGT reaction all plateaued at about 180 mP and the initial rates are approximately proportional with OGT concentrations, suggesting the success of the "FP-Tag" assay of OGT.

Large-scale HTSs are costly operations.[30] The per-well price of streptavidin during our "FP-Tag" HTS is less than 2 cents, leaving enough margin for other reagents, tips, and plates. However, streptavidin is still one of the major expenses during our large KpsC screening. Since streptavidin used in the "FP-Tag" assays merely served as a macromolecule, other proteins with relatively large molecular weight could potentially serve as a low-cost, alternative "FP-Tag", after introducing DBCO groups by *N*-hydroxysuccinimide (NHS) ester chemistry.

The first protein that came to our mind is the universally-available serum album (MW ~ 66 kDa), which has been used for FP study since the 1950s.[31-33] To test this idea, we incubated 10 eq. of DBCO-NHS with streptavidin (as a control), bovine serum albumin (BSA) and human serum albumin (HSA) for 1 h, and the excess of DBCO-NHS was removed by ultrafiltration. The resulting DBCO-SA, DBCO-BSA, and DBCO-HSA (10 μM based protein) conjugates were tested as "FP-Tags" by incubation with OGT reaction solution. Significant FP increases were observed for all three "FP-Tags" (Figure 3B). However, the background fluorescence of BSA and HSA is significantly higher, which leads to higher background FP signals.

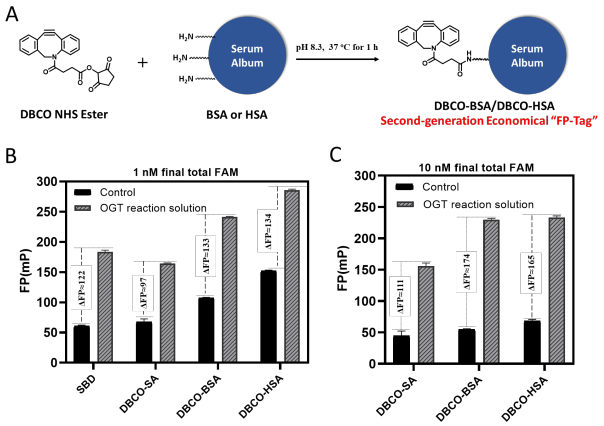

Figure 3. Evaluation of different "FP-Tags". A) Preparation of DBCO-BSA and DBCO-HSA conjugates. B) FP reading of SBD, DBCO-SA, DBCO-BSA, and DBCO-HAS as "FP-Tags" with OGT reaction (1 nM based on total FAM concentration). C) FP reading of DBCO-SA, DBCO-BSA, and DBCO-HSA as "FP-Tags" with OGT reaction (10 nM based on total FAM concentration). Each reaction was repeated three times. ΔFP refers to the FP increase of the experimental group relative to the control reaction without enzyme.

The background FP signals can be reduced by increasing the amount of the OGT assay solution (from 1 nM to 10 nM based on total final FAM concentration) transferred to the DBCO-BSA or DBCO-HSA solution as the fluorescence of FAM would become dominated in such solution (Figure 3C). We found DBCO-BSA and DBCO-HSA increase the FP by about 174 mP and 165 mP respectively. Interestingly, both BSA and HSA outperform streptavidin, either by direct DBCO linkage (111 mP) or DBCO-biotin linkage (122 mP). Those results suggest that serum album could serve as an effective and economical "FP-Tag". Since BSA is much cheaper than HSA, we subsequently focused our optimization effort on BSA.

We first investigated DBCO-NHS and BSA ratio by incubation of the corresponding DBCO-BSA conjugates with the OGT reaction solution. We found that increasing the ratio from 1/1 to 10/1, the ΔFP increases dramatically (Figure 4A). Increasing the DBCO-NHS ratio to 20 eq. and 40 eq. only gave comparable results, suggesting 10 eq. of DBCO is already enough to drive the click reaction to completion. Therefore, DBCO-NHS/BSA = 10/1 is chosen as the receipt to prepare the "FP-Tag".

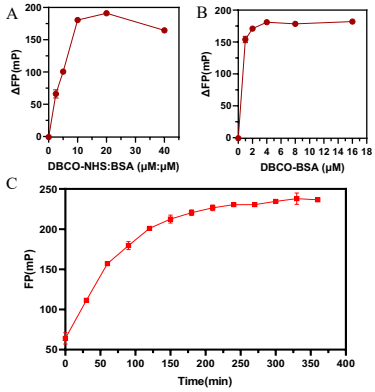

Figure 4. Optimization of DBCO-BSA based "FP-Tag" assay for OGT. A) The effect of DBCO-NHS and BSA ratio on ΔFP; B) The effect of DBCO-BSA concentration in the click reaction on ΔFP. Each experiment was repeated three times. ΔFP refers to the FP increase of the experimental group relative to the control group without enzyme. C) Time course of the OGT reaction progress monitored by DBCO-BSA based "FP-Tag" assay.

Secondly, we found the concentration of DBCO-BSA can be further reduced to 4 μM as it gave comparable FP reading with that of the higher concentrations (Figure 4B). Thus, the final DBCO-BSA concentration was set as 4 μM. With the optimized condition obtained, we retested the OGT reaction progress with DBCO-BSA as the "FP-Tag", and a similar but more responsive curve was obtained (Figure 4C), comparing to that of SBD.

As a proof of concept, a small scale of HTS of household marine natural product library (260 compounds) was performed in 96-well format. With DBCO-BSA as "FP-Tag", we obtained a Z' factor[34] to be 0.75. One known natural product, 2-methyleurotinone[35-36] was identified as a potential hit.

In our current FP assay, the FP signal is proportional to the reaction progress, therefore, the IC_{50} can potentially be obtained by plotting the FP signals with the inhibitor concentrations. Since FP reading is the ratio between the macromolecule-bounded fluorophore and free fluorophore, and the fluorescence behavior might be altered by attaching a fluorophore to a macromolecule, thus, it is unknown whether the FP signal is linear with the reaction conversion. Therefore, using the FP reading for IC_{50} determination could potentially be misleading.

In our case, the fluorophore will not disassociate from macromolecule because of the covalent linkage, thus the reaction conversion can be calculated from the FP reading by establishing an FP /conversion calibration curve. We proportionally mixed the full conversion reaction and no conversion reaction (reaction without OGT enzymes) and measured the FP reading of the corresponding mixed solutions. The proportion (same as reaction conversion) was then plotted with FP reading to get a calibration curve (Figure 5A). We found that the calibration curve is fairly linear with an R^2 about 0.99, suggesting macromolecule did not change the fluorescence of FAM. With the calibration curve in hand, the IC_{50} of 2-methyleurotinone was determined to be 11.9 μM with the "FP-Tag" assay (Figure 5B).

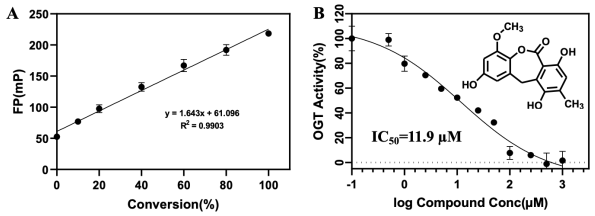

Figure 5. A) The FP/conversion calibration curve. B) The IC_{50} of 2-methyleurotinone, determined by the "FP-Tag" assay.

In summary, a high-throughput "FP-Tag" assay was developed for OGT. Although the previous-employed SBD is an effective "FP-Tag" in the OGT assay, we found DBCO-modified BSA is a superior alternative in terms of both performance and costs. The bulk-purchase price for streptavidin is about $3500/g, while that of BSA is only about $3/g, thus this economical "FP-Tag" dropped the cost by more than 1000 times, making the "FP-Tag" expense almost negligible during an HTS campaign. With this "FP-Tag" OGT assay, we screened a marine product library and identified a low μM-range OGT inhibitor.

Our "FP-Tag" assay format could be easily transferred to other glycosyltransferases (GTs) as only minimum modified sugar nucleotide analogs are needed. It is well-known that GT inhibitors are difficult to find, thus sometimes screening a large compound set is needed to get

suitable hits. Therefore, the BSA-based "FP-Tag" is well-suited for GTs that requires a large HTS campaign.

In addition, this kind of assay might find applications on the directed evolution of GTs. For example, a fluorophore can be attached to the acceptor sugar, after a mutant library of GT transferring an azide labeled sugar, DBCO-BSA is added to induced the FP increase, and the FP signal is proportional to the mutant GT activities. Therefore, we believe our "FP-Tag" assay format is a new direction of FP-based technology, and with BSA serves as low-cost "FP-Tag", this technique would find more board applications.

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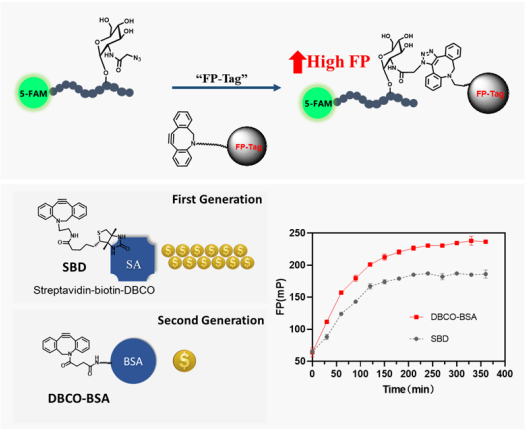
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High FP
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First Generation
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SA
Streptavidin-biotin-DBCO
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DBCO-BSA
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