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Supporting Information

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Nanoparticle-based Energy Transfer for a Rapid and Simple Detection of Protein Glycosylation

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Synthesis of AuNPs

AuNPs were synthesized using a slightly modified citrate reduction and stabilization method as described elsewhere.[22] Briefly, 10 mg of $HAuCl_4 \cdot 3H_2O$ (Aldrich) was dissolved in 100 mL of distilled water and vigorously stirred for 1 min followed by the addition of 20 mg of sodium citrate dehydrate (2-hydroxy-1, 2, 3-propanetricarboxylic acid) and stirring. After 1 min, 85 μL of stock solution containing 11.4 mg of $NaBH_4$ in 1mL DDW was added quickly to the reaction solution for the reduction and formation of gold colloids, and stirred for 5 min. The completely reduced solution containing 254 μM of Au in total was stored at 4°C for conservation.

Conjugation of concanavalin A (ConA) to AuNPs

One milliliter of citrate stabilized AuNPs was added to 1 mL DDW containing 50 μM ConA followed by mild stirring for 8 h. It is known that effective conjugation of ConA to AuNPs through physical adsorption occurs at slightly basic condition against the isoelectric point of ConA (PI = 4.5 ~ 5.1, information from supplier). Thus, the conjugation was carried out at pH 6.0 The resulting mixture was filtered through a microfilter with the molecular weight cut-off of 100 kD (Microcon YM100, Millipore Corp.) in order to remove the free ConA. Following a washing step with 10 mM Tris-HCl buffer (pH 3.0) five times, ConA-conjugated AuNPs (ConA-AuNPs) were resuspended to 10 mM DDW containing 1.3 mM Ca^{2+} and 1 mM Mn^{2+}, respectively. Acidic pH prevents ConA from being assembled to tetramer, leading to an effective washing. Resultantly the number of ConA conjugated per AuNP was estimated to be 3 ~ 4 according to the Bradford dye assay, when considering its size of 3.2 nm and ~ 1000 Au atoms per AuNP.[23] Thus, concentrations of AuNP and ConA on the ConA-AuNPs contained in each assay solution corresponded to about 2.5 nM and 10 nM, respectively.

Conjugation of dextran to QDs

Hydroxyl groups of dextran were activated using 1,1'-Carbonyldiimidazole (CDI) as described elsewhere.[24] In brief, 200 μL of stock solution composed of 10 mg CDI in 500 μL of dimethylsulfoxide (DMSO) was added to 500 μL of 10 mM dextran solution in DDW. Following a reaction for 30 min at 37°C, 4.8 μL of CDI modified dextran solution was added to 0.95 ml of 80 nM amine-terminated QDs in DDW, and placed for 24 h at room temperature with mild stirring. The resulting mixture was filtered and washed four times with DDW through a microfilter with the molecular weight cut-off of 50 kD (Microcon YM50, Millipore Corp.) in order to remove the free dextran and CDI. The dextran-conjugated QDs (Dex-QDs) was resuspended in DDW and stored in dark at 4°C before use.

Strains, plasmids, and culture media for production of recombinant GOx

S. cerevisiae strain L3262a (*MATa ura3-52 leu2-3, 112 his4-34*) harboring pYGOx-His and *H. polymorpha* strainDL1-L (*leu2*) having pDLMOX-GOx(H) [20] were used for production of recombinant GOx tagged with 6 histidine residues, respectively. *H. polymorpha* glycosylation defective mutant strain *Hpalg3?* (*leu2, alg3::URA3*) was constructed from *H. polymorpha* DL1-LdU (*leu2, ura3*) *by* deleting the *HpALG3* gene through a gene disruption technique, in which the coding region of *HpALG3* was removed and then replaced with the *HpURA3* blaster cassette.[21] The resulting variant *Hpalg3?* was subjected to transformation with pDLMOX-GOx(H) to produce hypomannosylated GOx.

Production and purification of GOx

His_6-tagged recombinant GOx (rGOx) was produced by cultivation of yeast host cells harboring recombinant plasmids in a 250 mL flask at 30°C for *S. cerevisiae* and 37°C for *H. polymorpha*. The culture conditions to induce the expression of rGOx from the *GAL10* promoter or the *MOX* promoter were described previously.[20-21] His_6-tagged rGOx was purified by using Ni^{2+}-nitrilotriacetic acid (Ni-NTA) metal affinity resin (QIAGEN Inc.) as described elsewhere.[20] Culture supernatant containing rGOx was concentrated by ultra filtration followed by dialysis against 50 mM sodium phosphate buffer (pH 6.0) containing 300 mM NaCl. The resulting solution was applied to a Ni-NTA column pre-equilibrated with the same buffer. The column was washed with a buffer, and His_6-tagged rGOx was eluted with an imidazole gradient up to 500 mM by using a ÄKTA prime chromatography system (Amersham Pharmacia Biotech AB). The purified rGOxs were used for further experiments after dialysis against the appropriate buffer. The electrophoretic mobility of rGOxs and authentic GOx from *A. niger* (Sigma-Aldrich) were analyzed on SDS-polyacrylamide gels before and after treatment with PNGase F (New England Biolabs) according to the manufacturer's instruction.

Synthesis of neoglycoprotein

BSA is known to have 60 lysine residues, and a portion of surface lysine residues of BSA were modified with a-D-mannopyranosyl-phenyl-isothiocyanate (MPI) through covalent bonding between isothiocyanate and e-amine group.[25] An MPI stock solution was freshly prepared in DMSO, and the coupling reaction was carried out in a 0.05 M sodium bicarbonate buffer (pH 9.0) containing 20% DMSO in total at 4ºC for 24 h. For the synthesis of the BSA having different mannosylation degrees, the molar ratio of MPI to BSA varied from 1.5 to 150. The resulting mixture was filtered and washed four times with a 10 mM sodium bicarbonate buffer through a microfilter with the molecular weight cut-off of 10 kD (Microcon YM10, Millipore Corp.) in order to remove the free MPI. The purified, mannosylated BSA (Man-BSA) was subjected to carbohydrate analysis as described below, and was resuspended in the 10 mM sodium bicarbonate buffer and stored in -10ºC for conservation.

Preparation of Apo-GOx

The preparation of apo-GOx was followed as described elsewhere. [26] Purified GOx was dissolved in 500 µL of 40 mM sodium acetate buffer, and 500 µL of an ammonium sulfate ($(NH_4)_2SO_4$, 25% saturated, pH 1.4) solution was added. Then the sample was incubated in an ice bath with mild stirring for 2 h. Excess ammonium sulfate was added to the sample to separate FAD cofactor from GOx. The protein without FAD cofactor (apo-GOx) was pooled through a microfilter with the molecular weight cut-off of 10 kD (Microcon YM10, Millipore Corp.), washed five times, and resuspended in DDW. The resulting apo-GOx showed no yellow color, and lost its own fluorescence from FAD at 510~520 nm when excited at 440 ~ 460 nm (data not shown).

Analysis of neoglycoprotein

The number of mannoses in mannosylated BSA was analyzed by using Bio-LC DX-600 (Dionex, USA) Briefly, 10 µg of purified neoglycoprotein was reacted with 400 µL of 2 M trifluoroacetic acid at 100ºC for 4 h and freeze dried. The resulting sample was dissolved in 100 µL of DDW, diluted when necessary, and analyzed by using Column CarboPac PA1 (4.5 × 250 mm, Dionex, USA) equipped with CarboPac PA1 cartridge (4.5 × 50 mm). The injection volume was 10 µL, and mobile phase was 16 mM NaOH. The flow rate was 1.0 mL/min during analysis. The signal was detected by ED50 with integrated amperometry.

Analysis of *N*-linked oligosaccharides

The profiling of *N*-linked oligosaccharides was carried out by using size-fractionation HPLC as previously described.[20] Briefly, oligosaccharides were released from ~200 µg of purified rGOx or authentic GOx by treatment with PNGase F, and labeled with 2-aminopyridine (PA) using a commercially available reagent kit (Takara Shuzo Co.). Following a pyridylamination, the sample was purified again using a Sephadex G-15 spin column (Amersham Pharmacia Biotech AB) to remove the

residual PA. Size-fractionation HPLC was performed using a Shodex Asahipak NH2P-50 column (Showa Denko K. K.) using a chromatography system (Waters 247). PA-oligosaccharides were detected by fluorescence (λ_{ex} = 320 nm and λ_{em} = 400 nm) using a fluorescence detector (Waters 2475).

Four-parameter logistic equation

The four-parameter logistic equation used for fitting of sigmoid dose-response data is as follows;

$$Y = \frac{A_1 - A_2}{1 + (X/X_0)^p} + A_2$$

where A_1 = initial Y value, A_2 = final Y value, X_0 = X value when $Y(X_0) = (A_1 + A_2) / 2$, and p = power. Accordingly, IC_{50} is defined as X_0.

Normalized PL differences shown in Fig. 2 were fit to the above equation with respect to the protein concentration. As a result, the following parameters were determined for avidin and 22-MB, respectively.
For avidin, A_1 = - 0.010, A_2 = 1.026, X_0 = 8.528 μM, and p = 1.646.
For 22-MB, A_1 = - 0.032, A_2 = 1.020, X_0 = 0.120 μM, and p = 0.912.

Table S1. Analysis of neoglycosylated BSA. Number of mannoses per BSA was analyzed by using Bio-LC DX-600.

Man-BSA	No. mannose / BSA
22-MB	22.0
21-MB	21.4
15-MB	15.0
13-MB	12.6
10-MB	10.0
8-MB	8.2
6-MB	5.7
3-MB	2.8
2-MB	1.5
0-MB(BSA)	0

Fig. S1. Characteristics of Con A - AuNPs. (a) EF-TEM image of ConA-AuNPs. ConA-AuNPs have a spherical shape with an average diameter of 3.2 ± 0.4 nm. An image was obtained with EF-TEM (EF-TEM, EM912 Omega, Carl Zeiss, Germany). The average size of the AuNPs was estimated from the TEM image. For a clearer image, a 300 mesh carbon TEM grid was used. About 3 ~ 5 μL of each sample solution was dropped on the grid and dried at room temperature. (b) Absorbance spectra of AuNPs (dotted line) and ConA-AuNPs (solid red line). AuNPs shows the surface plasmon band at around 510 nm. Meanwhile, ConA-AuNPs displays broader and more red-shifted spectrum. The red-shifted absorption spectrum with a peak at 523 nm indicates the protein conjugation on the surface of AuNPs. The TEM image of ConA-AuNPs revealed the uniform size distribution of AuNPs even after ConA conjugation and no significant aggregation among ConA-AuNPs.

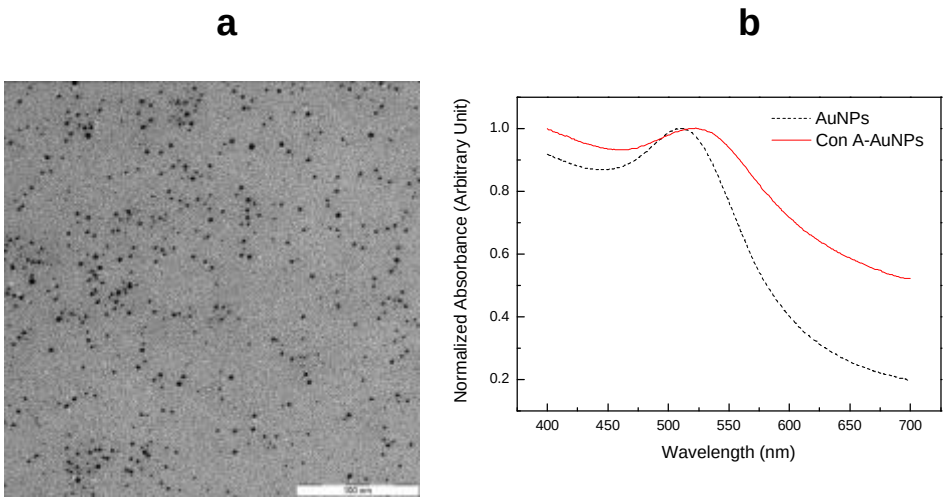


Fig. S2. PL quenching of Dex-QDs by ConA-AuNPs. (a) Normalized PL quenching of 400 pM of Dex-QDs as a function of ConA-AuNPs concentration in assay solution. PL quenching (? P) was normalized against the maximum PL quenching (? P_{max}) which was registered when the concentration of ConA on the ConA-AuNPs was about 60 nM. Scale bars represent the standard deviation in triplicate experiments. The PL intensity of Dex-QDs was measured at 605 nm. The photoluminescence (PL) quenching of Dex-QDs at 605 nm increased as more ConA-AuNPs were added, resulting in a semi-logarithmic correlation with the concentration of ConA-AuNPs. (b) The plot for determination of the apparent association constant, K_a, between Dex-QDs and ConA-AuNPs. [ConA-AuNPs] P_0/? P_{max} was plotted against [ConA-AuNPs] P_0/? P to estimate the K_a from the intercept according to the following equation: [1]

$$\frac{[\text{ConA}-\text{AuNPs}]P_0}{?\,P} = \frac{[\text{ConA}-\text{AuNPs}]P_0}{?\,P_{max}} + \frac{P_0}{?\,P_{max}K_a}$$

where P_0 and P are the PL intensities of the Dex-QDs before and after the addition of ConA-AuNPs, respectively. P_{max} is the PL of Dex-QDs when maximum quenching occurs by ConA-AuNPs. Accordingly, ? P = P_0 - P and ? P_{max} = P_0 - P_{max}. From this result, the apparent binding constant (K_a) between ConA-AuNPs and Dex-QDs was calculated to be 6.7 ×10^7 M^{-1}.

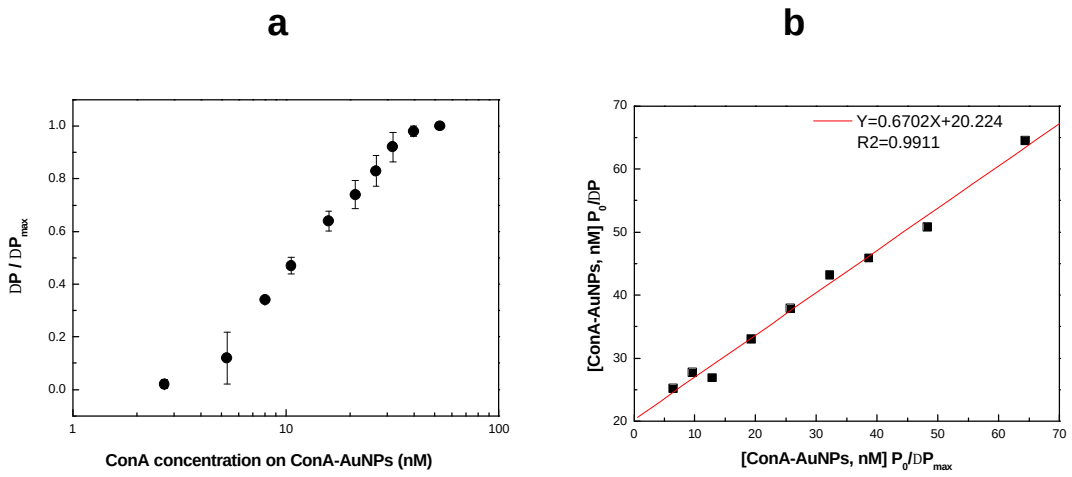


Fig. S3. SDS-PAGE analysis of recombinant and authentic GOxs. Recombinant GOxs (rGOxs) produced by *S. cerevisiae* (lane 1 and 5), *H. polymorpha* (lane 2 and 6), *Hpalg3?* (lane 3 and 7) strains, and an authentic one from *A. niger* (lane 4 and 8) were purified using Ni-NTA affinity resin followed by an analysis on SDS-PAGE. Lanes 5 ~ 8 indicate the GOxs treated with PNGase F.

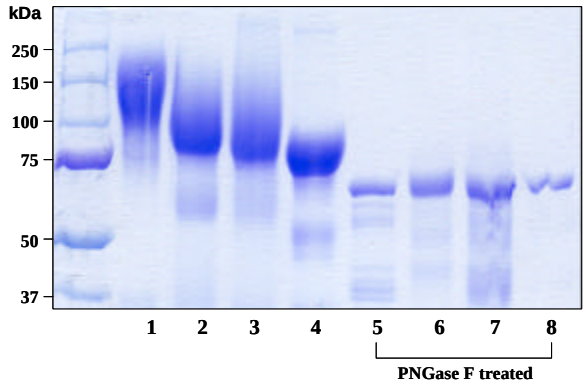
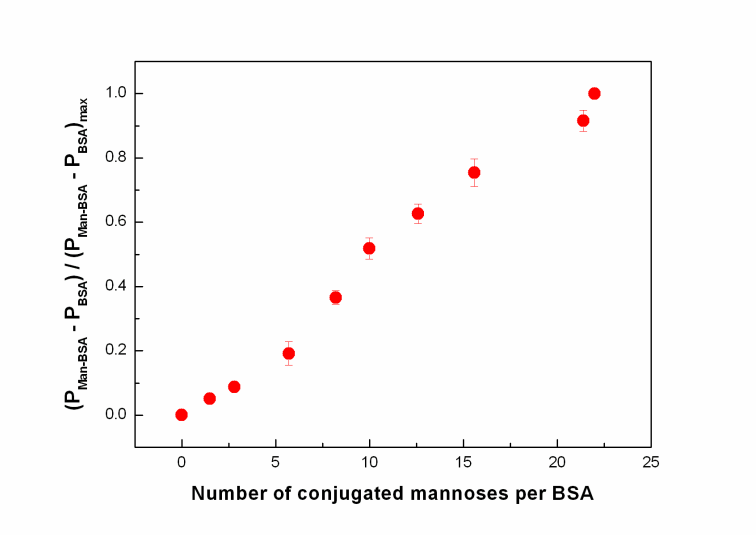


Fig. S4. Detection of diversely mannosylated BSAs by using a luminescent image analyzer. The PL intensities from pixels in the image spot of each well plate were averaged and used for data analysis. Normalized intensity differences between mannosylated BSA and native BSA, $(P_{Man\text{-}BSA}\text{-}P_{BSA})/(P_{Man\text{-}BSA}\text{-}P_{BSA})_{max}$, were plotted against the number of mannoses conjugated to BSA. Scale bars indicate the standard deviation in triplicate experiments.



References

[1] T. Hasegawa, S. Kondoh, K. Matsuura, K. Kobayashi, *Macromolecules* **1999**, *32*, 6569-6603.