Protein Analysis

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Nanoparticle-Based Energy Transfer for Rapid and Simple Detection of Protein Glycosylation**

*Eunkeu Oh, Dohoon Lee, Young-Pil Kim, Seung Youp Cha, Doo-Beyong Oh, Hyun Ah Kang, Jungbae Kim, and Hak-Sung Kim**

Glycosylation is one of the most important posttranslational modifications of proteins. The specific oligosaccharide structure or moiety at each glycosylation site plays an essential role in determining the properties of the glycoprotein, including in vivo biological activity, solubility, protease resistance, immunogenicity, signal transduction, and pharmacokinetics.[1–4] The development of protein therapeutics requires stringent control of the glycosylation type and the degree of glycosylation in the target protein. These properties can be controlled by modulating the operating parameters, such as the glycan-processing enzymes, culture conditions, and host strains, in the production of the target protein.[5] In this regard, the structural or quantitative analysis of oligosaccharides is of great importance in terms of both practical and fundamental aspects. However, most conventional analytical methods are based on mass spectrometry and chromatography, which require complex and laborious sample preparation, such as deglycosylation.[1]

Approaches based on nanoparticles (NPs) have drawn much attention as more efficient methods for the detection of biomolecules because of their size-dependent physical and chemical characteristics.[6–15] Recently, the signal quenching

[*] Dr. E. Oh,[+] Dr. D. Lee, Y. P. Kim, Prof. H. S. Kim
Department of Biological Sciences
Korea Advanced Institute of Science and Technology
Daejeon, 305-701 (Korea)
Fax: (+82) 42-869-2610
E-mail: hskim76@kaist.ac.kr

S. Y. Cha, Dr. D. B. Oh, Dr. H. A. Kang
Protein Therapeutics Center
Korea Research Institute of Bioscience and Biotechnology
Daejeon, 305-600 (Korea)

Dr. J. Kim
Pacific Northwest National Laboratory
902 Battelle Boulevard, P.O. Box 999, Richland, WA 99352 (USA)

[+] Current address:
Samsung SDI Co., 428-5 Gongse-dong, Giheung-gu
Yongin-si, Gyeongggi-do, 446-577 (Korea)

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due to the energy transfer between a quantum dot (QD) and a gold nanoparticle (AuNP) in close proximity was investigated for use in biosensing, owing to its speed, simplicity, and even sensitivity.[14,15] Several mechanisms have been proposed to explain this quenching phenomenon and investigated rigorously, such as fluorescence resonance energy transfer (FRET)[13,14] and surface energy transfer (SET).[11] Herein, we describe an approach for the rapid and simple detection of protein glycosylation based on the energy transfer between lectin-conjugated AuNPs and carbohydrate-conjugated QDs (Figure 1). We also demonstrate the potential of our system in the high-throughput analysis of glycosylation degree, which is critical for the development of protein therapeutics.

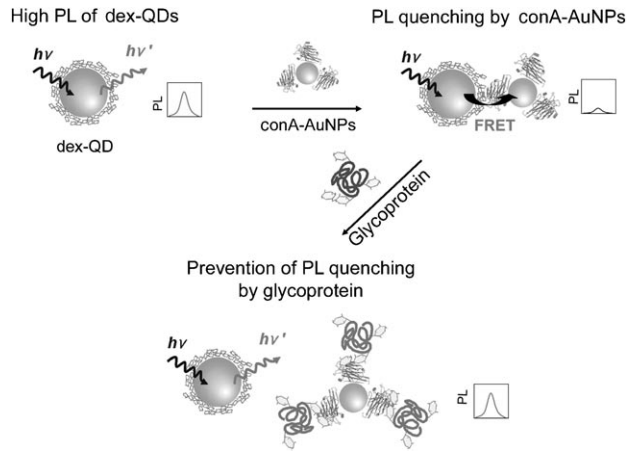

Figure 1. NP-based energy transfer for the detection of protein glycosylation. Top left: free dex-QDs; top right: dex-QDs and conA-AuNPs; bottom: the addition of a glycoprotein prevents PL quenching of dex-QDs by inhibiting the association between dex-QDs and conA-AuNPs, and thus restores the degree of PL of the dex-QDs to that of free dex-QDs.

For the construction of an NP-based detection system, AuNPs were conjugated with concanavalin A (conA), which is a lectin with preferred binding affinity to manno- and glucooligosaccharides (see the Supporting Information),[16] and QDs were conjugated with dextran, which is a polymeric form of glucose. We examined the binding of conA-conjugated AuNPs (conA-AuNPs) to dextran-conjugated QDs (dex-QDs) by measuring the emission spectrum of the dex-QDs. The photoluminescence (PL) quenching of dex-QDs at 605 nm increased as more conA-AuNPs were added. The apparent binding constant (K_a) of $6.7 \times 10^7\,\text{M}^{-1}$ between conA-AuNPs and dex-QDs calculated from this result (see the Supporting Information) is similar to the previously reported value of $1.1 \times 10^7\,\text{M}^{-1}$,[17] which confirms that the PL quenching of dex-QDs occurs specifically by conA-AuNPs. We also checked whether the PL quenching can be prevented by a protein that inhibits the interaction between conA-AuNPs and dex-QDs. The PL intensity of the dex-QDs was quenched by about 70% in the presence of conA-AuNPs (Figure 2). Upon the addition of free conA, the PL recovered to almost the same level of that of free dex-QDs, thus indicating that the free conA added inhibited the binding of conA-AuNPs to dex-QDs. These results suggested that this system could be

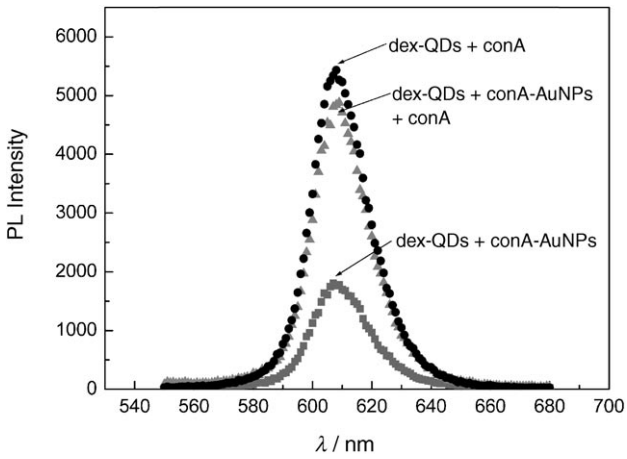

Figure 2. Inhibition assay with dex-QDs and conA-AuNPs. PL of free dex-QDs: ●; PL quenching of dex-QDs by conA-AuNPs: ■; PL quenching was inhibited by the addition of conA (5 μM): ▲.

used for the study of protein glycosylation as depicted in Figure 1.

To demonstrate the validity of this concept, we tested whether the system can distinguish the presence of a glycan moiety on intact proteins. As proposed above, the detection of a glycoprotein relies on a change in the efficiency of energy transfer between conA-AuNPs and dex-QDs; the glycan moiety on the glycoprotein caused this change by inhibition. Avidin and its deglycosylated counterpart neutravidin (Pierce, Rockford, USA) were employed as model proteins. Each subunit of avidin has one potential glycosylation site and about four mannose residues.[18] The PL intensity of the dex-QDs was measured after the addition of each protein. In the case of avidin, the PL intensity at 605 nm, PL_A, increased as the avidin concentration increased up to 100 μM. In contrast, the PL intensity of neutravidin, PL_N, displayed no concentration dependency (data not shown). This result indicates that avidin, which has a glycan moiety, acts as a competitive inhibitor of the binding of dex-QDs to the conA of conA-AuNPs, but neutravidin does not. The PL difference between avidin and neutravidin was normalized against the maximum PL difference at excess protein concentration. As a result, the normalized PL difference, $(PL_A - PL_N)/(PL_A - PL_N)_{max}$, increased with a logarithmic increase in protein concentration (Figure 3). By fitting the data to the four-parameter logistic equation (see the Supporting Information), the IC_{50} value (the protein concentration at which the normalized PL difference is half its maximal value) of avidin was estimated to be 8.5 μM. In another test with bovine serum albumin (BSA) and its chemically neoglycosylated form 22-MB (BSA–α-D-mannopyranosylphenyl isothiocyanate with 22 mannose units per molecule of BSA) a similar trend was observed (Figure 3). However, the IC_{50} value of 22-MB was thus estimated to be 120 nM, two orders of magnitude lower than that of avidin. This result indicates that 22-MB has many more mannose residues and a higher inhibition efficiency than avidin.

This system we had developed was applied to the analysis of proteins with varying degrees of glycosylation. For this purpose, mannose residues were conjugated chemically to the

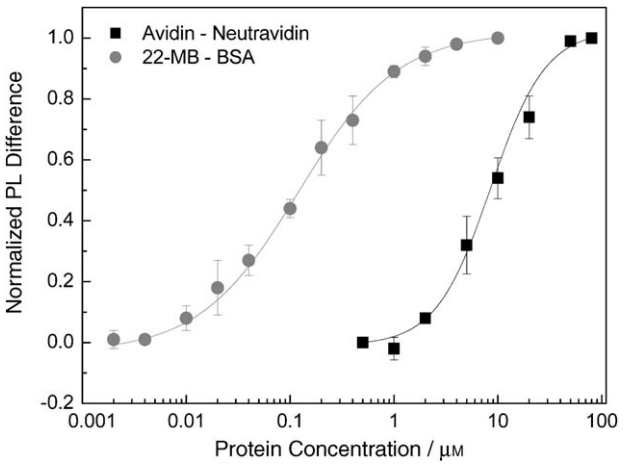

Figure 3. Detection of the glycan moiety of different glycoproteins. Normalized PL differences for avidin–neutravidin (■), $(PL_A-PL_N)/(PL_A-PL_N)_{max}$, and for 22-MB–BSA (●), $(PL_{22\text{-}MB}-PL_{BSA})/(PL_{22\text{-}MB}-PL_{BSA})_{max}$. The solid lines were obtained by fitting the four-parameter logistic equation to the data. Scale bars correspond to the standard deviation in triplicate experiments. In both curves, R^2 values are larger than 0.99.

BSA in different molar ratios. The mannose moiety of α-D-mannopyranosylphenyl isothiocyanate (MPI) was coupled with the lysine residue of the BSA through a covalent bond. The resulting differently mannosylated BSA molecules (Man-BSAs) were analyzed to quantify the number of conjugated mannose units per BSA molecule by using BioLC chromatography (see the Supporting Information). After the addition of each Man-BSA to the assay solution containing dex-QDs and conA-AuNPs we measured PL. The emission spectrum of the dex-QDs changed with an increasing number of mannose moieties on the BSA (Figure 4a), and the normalized PL difference between Man-BSAs and the native BSA at 605 nm, $(PL_{Man\text{-}BSA}-PL_{BSA})/(PL_{Man\text{-}BSA}-PL_{BSA})_{max}$, was enhanced as the occupancy number of the mannosylation sites increased (Figure 4b). These results illustrate that this system can be used effectively for the analysis of the degree of glycosylation of proteins.

In an attempt to expand the utility of our detection system, we tested glycoproteins with different glycan profiles. First, three kinds of recombinant glucose oxidases (rGOxs) with different lengths of high-mannose-type glycans were produced from the yeast strains *Saccharomyces cerevisiae* (*S. cerevisiae*), *Hansenula polymorpha* (*H. polymorpha*), and the glycosylation-defective mutant strain *Hpalg3*Δ of *H. polymorpha*. The GOx from *Aspergillus niger* (*A. niger*) was also tested as the authentic glycoprotein. The HPLC profile analysis of N-linked oligosaccharides clearly revealed that the GOx of each host strain has a distinct glycan profile, that is, a different distribution of mannose chains attached to the GOx (Figure 5a and the Supporting Information). As shown in Figure 5b, the responsive PL intensity of the assay with dex-QDs and conA-AuNPs varied distinctly with respect to the degree of mannosylation of the GOx. The hypermannosylated GOx from *S. cerevisiae* exhibited the highest PL intensity, which indicates that this GOx inhibited the PL quenching by conA-AuNPs most effectively. As expected, the

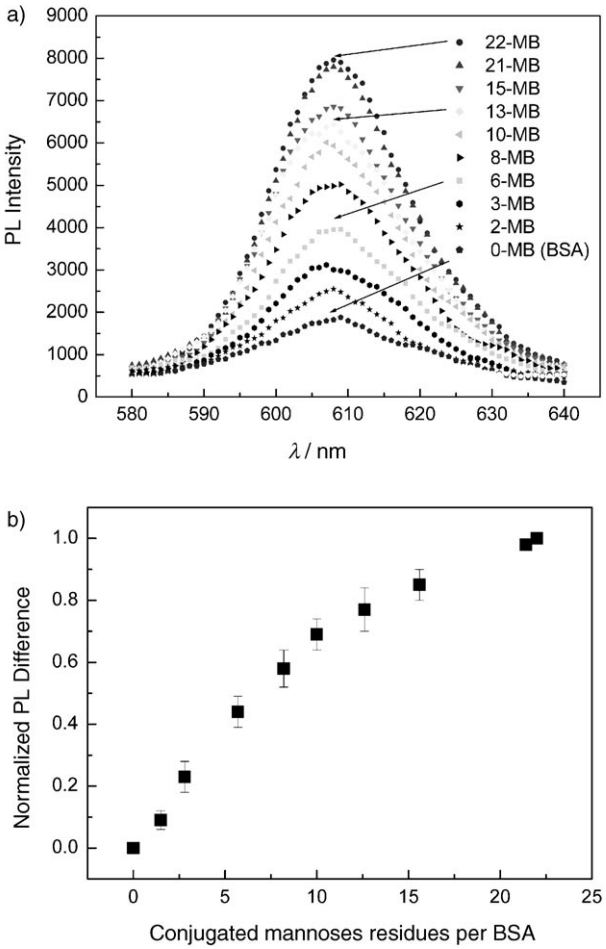

Figure 4. Changes in PL quenching of dex-QDs in the presence of diversely mannosylated BSA. a) Change in the emission spectra of dex-QDs caused by various Man-BSAs. The number in front of -MB indicates the number of conjugated mannose residues per BSA molecule. b) Normalized PL differences between Man-BSAs and native BSA, $(PL_{Man\text{-}BSA}-PL_{BSA})/(PL_{Man\text{-}BSA}-PL_{BSA})_{max}$. Scale bars indicate the standard deviation in quadruplicate experiments. The Man-BSA concentration was 1 μM, at which the inhibition effect by 22-MB was sufficient but smaller than that at saturation (see the Supporting Information).

hypomannosylated GOx from *Hpalg3*Δ gave rise to the lowest PL intensity. On the basis of these observations, it is evident that the PL intensity correlates well with the length of branched mannose chains on the proteins. Thus, these results clearly demonstrate the utility of our detection system.

To examine the possibility of high-throughput analysis of the degree of glycosylation, we conducted parallel detection of Man-BSAs by using a miniwell plate. The protein amount and the total volume of each assay were 556 ng and 5 μL, respectively. The PL intensity from the well plate was measured with a luminescent image analyzer (Figure 6), and the normalized PL difference increased with an increasing number of occupied mannosylation sites (see the Supporting Information). This is a similar trend to that observed for the PL described in Figure 4b.

We have demonstrated that NP-based energy transfer can be used effectively for the detection of glycan moieties and

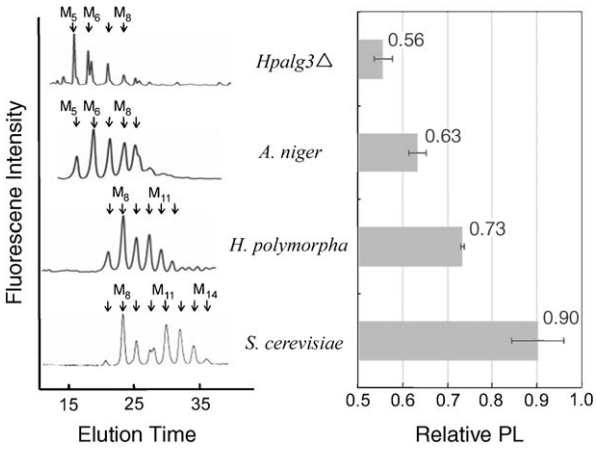

Figure 5. Detection of GOxs with varied glycan profiles. a) Glycan profiles of N-linked oligosaccharides released from GOxs were analyzed by HPLC. The length distribution of the mannose chains was assigned by comparison of the elution time with those of the reference oligosaccharides $Man_nGlcNAc_2$-PA (M_n; $n = 5, 6, 8, 11, 14$), which are indicated by arrows. The GOx produced from *Hpalg3*Δ contained the shortest chains with a distribution of mainly five to seven mannose residues. GOx produced by wild-type *S. cerevisiae* exhibited the longest chains (ranging from 8 to 14 residues). GOxs from wild-type *H. polymorpha* and *A. niger* were found to be mainly composed of 8–11 and 6–9 residues, respectively. b) Relative PL intensity of dex-QDs in the presence of the GOx. The assay was conducted by using apo-GOxs to eliminate the signal interference from flavin adenine dinucleotide coenzyme. GOx concentration: 75 nM.

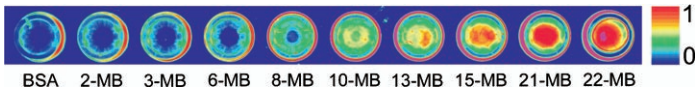

Figure 6. Pseudocolor image created by a luminescent image analyzer of a miniwell plate containing Man-BSAs and the assay solution. The number in front of -MB indicates the number of conjugated mannose residues per BSA molecule. A warmer color indicates a higher intensity at 605 nm.

the degree of glycosylation of proteins. This rapid and simple approach provides a reliable assay of protein glycosylation without tedious traditional deglycosylation and labeling steps. Only tiny amounts of glycoprotein and NPs are required for the completion of an assay because of high PL intensity and negligible bleaching of QDs. Furthermore, the dynamic range and sensitivity of this system can be modulated or extended readily by controlling the size, surface properties, and quantity of the NPs, as well as the number of conjugated molecules on the NPs. This system involving NP-based energy transfer could be implemented for multiplexed detection by using combinations of different lectins and QDs, and could contribute to a high-throughput analytical method for the development of glycoprotein and carbohydrate therapeutics. We also expect to be able to determine the complex glycan profile of a glycoprotein by employing a variety of lectins.

Experimental Section

Production of rGOxs: GOx from *A. niger* has eight potential sites for N-linked glycosylation.[19] rGOxs from other hosts were prepared by previously reported methods (Supporting Information).[20,21]

Synthesis of conA-AuNPs: AuNPs of 3.2 nm in size were synthesized and conjugated with conA (type IV from *Canavalia ensiformis*, Sigma) by direct adsorption (see the Supporting Information).[22] The number of conjugated conA molecules per AuNP was estimated to be three or four by considering the size of the AuNPs, each of which contains approximately 1000 Au atoms.[23]

Synthesis of dex-QDs: Amine-functionalized QDs (rod shaped; diameter ≈5 nm, length 10–15 nm, Quantum Dot Corporation) with an emission peak at 605 nm were conjugated to dextran activated with 1,1′-carbonyldiimidazole (see the Supporting Information).[24]

Synthesis of neoglycoprotein: MPI was used for the conjugation of mannose to BSA according to the reported method (see the Supporting Information).[25]

Preparation of apo-Gox: Apo-GOx was prepared by using ammonium sulfate as described elsewhere (see the Supporting Information).[26]

Detection of PL: The emission spectrum and PL intensity at 605 nm of dex-QDs were measured after excitation at 440 nm by a PL spectrometer (SLM-AMINCO). The assay solution was composed of conA-AuNPs (2.5 nM), dex-QDs (200 pM), Ca^{2+} (1.3 mM), Mn^{2+} (1 mM), and Mg^{2+} (1 mM) in doubly distilled water (200 μL).

Parallel analysis in miniwell plates: The CCD image of a miniwell plate containing dex-QDs was created and analyzed by a luminescent image analyzer (LAS-3000, Fujifilm, Japan, excitation at 460 nm, emission at 605 nm). The assay solution was composed of conA-AuNPs (4.17 nM), dex-QDs (333 pM), Man-BSA (1.67 μM, 556 ng), Ca^{2+} (1.3 mM), Mn^{2+} (1 mM), and Mg^{2+} (1 mM) in doubly distilled water (5 μL).

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