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Liberation of Protein-Specific Glycosylation Information for Glycan Analysis by Exonuclease III-Aided Recycling Hybridization

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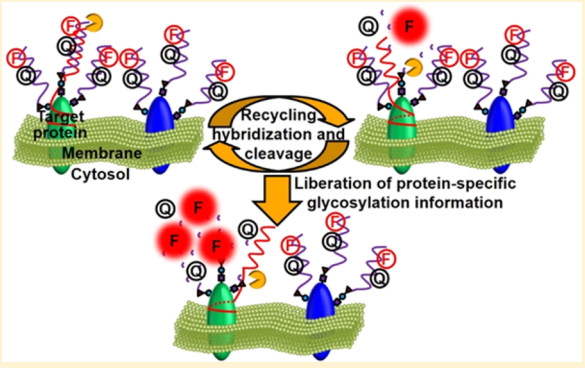
Supporting Information

ABSTRACT: A strategy for information liberation of protein-specific glycosylation is designed via an exonuclease III-aided recycling "hybridization and cleavage" process with glycan and protein probes, which achieves homogeneous quantification of cell surface glycan. The protein probe contains matching and spacer DNA sequences and an aptamer specific to target protein. The glycan probe contains a complementary sequence modified with neighboring fluorescein and quencher, a spacer sequence, and a dibenzocyclooctyne-amine end to bind azide-tagged glycan. Upon sequential binding to their targets, the complementary sequences of two probes approach enough for their hybridization, which leads to the cleavage of hybridized glycan probe by exonuclease III and followed recycling "hybridization and cleavage" process of protein probe with other adjacent glycan probes to release the labeled fluorescein for obtaining the information on protein-specific glycosylation. This protocol has been used to in situ quantify EpCAM-specific sialic acid on MCF-7 cell surface and monitor its variation during drug treatment. This work demonstrates a powerful quantification tool for research of glycosylation.



Glycosylation of cell surface protein can affect cellular adhesion or motility, which further reflects the physiological and pathological states of cells.[1−3] So precise quantitative information on protein-specific glycosylation is important for uncovering its role in disease development and diagnosis. Some analytical methods based on Förster resonance energy transfer (FRET) have been developed for protein-specific imaging of glycans.[4−6] These methods usually use two FRET-achievable fluorescent molecules to label protein and the corresponding glycan, respectively, which can bring these fluorescent molecules close under a certain distance to achieve FRET for visualization. Several mass spectrometric strategies combined with enzyme-linked immunosorbent assay (ELISA) have also provided protein-specific glycan information at the molecular level.[7−9] However, the quantitative signal, particularly in situ detection, of protein-specific glycan is still a challenge and urgent demand.

This work designs two DNA probes, protein probe (PP) and glycan probe (GP), for in situ recognition of target protein and binding of its specific glycan on the cell surface (Figure 1). The PP is composed of matching and spacer DNA sequences and an aptamer specific to target protein. The GP contains an oligonucleotide sequence complementary to the matching sequence of PP and modified with neighboring fluorescein (6-carboxyfluorescein, FAM) and quencher (black hole quencher, BHQ) and a dibenzocyclooctyne-amine (DIBO) at 5′ end of the spacer sequence for specifically binding the azide group through copper-free click chemistry.[10−12] After PP and GP sequentially bind to target glycoprotein and the specific glycan, respectively, PP can hybridize with an adjacent GP to form a duplex DNA in the hybridization buffer, which can be cleaved by exonuclease III (Exo III) to remove the mononucleotides from the 3′-hydroxyl termini of GP[13] and release the labeled fluorescent molecule. The left PP can further hybridize with other adjacent GPs to achieve recycling hybridization and cleavage. Owing to the spacer length of around 2 nm for octadecane, the efficient distance between hybridizable PP and GP is limited at 4 nm, which excludes the hybridization possibility of PP with the GP bound on non-protein-specific glycan. Thus, the released fluorescent molecule is related to all azide-tagged glycan bound on the target protein. With a metabolic labeling technology to introduce an azide group to the glycan,[14−16] the fluorescent intensity can represent the signal of protein-specific glycan on the whole cell surface when the incompleteness of metabolic labeling can be ignored, which leads to a simple and homogeneous protocol for

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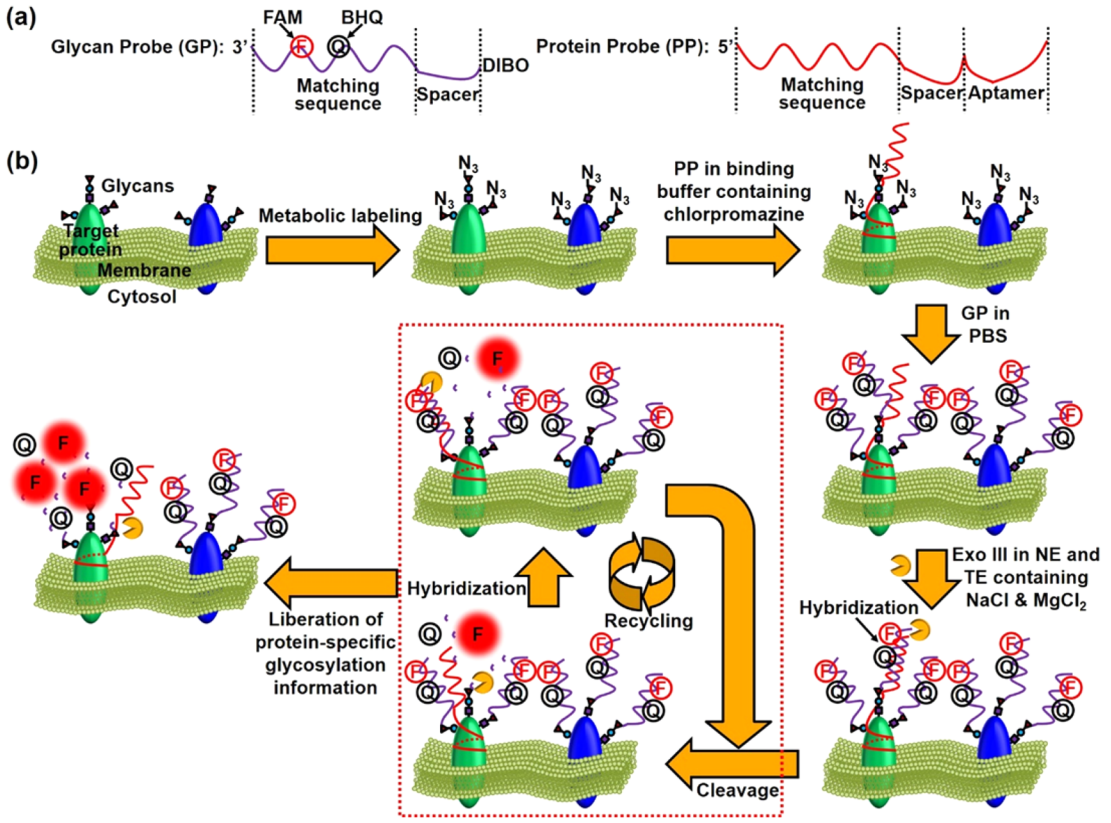

Figure 1. Schematic illustration of (a) two DNA probes and (b) Exo III-aided recycling hybridization to liberate the information on protein-specific glycosylation on the cell surface.

quantitatively revealing the glycosylation level of the target protein.

As a proof of concept, epithelial cell adhesion molecule (EpCAM) was used as the target protein, which can mediate epithelial-specific intercellular cell adhesion, signaling, migration, proliferation, and differentiation[17,18] and highly expresses on human breast cancer MCF-7 cell membrane.[19] It contains three potential N-linked glycosylation sites but no O-linked glycosylation site.[20] Thus, cell surface sialic acid (Sia) was selected as the target-specific glycan to perform metabolic labeling with tetraacetylated *N*-azidoacetyl-D-mannosamine (ManNAz), while tetraacetylated *N*-azidoacetylgalactosamine (GalNAz) was used to metabolically label cell surface O-linked glycans (OLG)[21,22] as negative control. The designed strategy successfully achieved the in situ quantification of EpCAM-specific sialic acid on MCF-7 cell surface and monitored its variation during drug treatment, which demonstrates a powerful quantification tool for uncovering glycosylation-related biological processes at the protein-specific level.

EXPERIMENTAL SECTION

Materials and Reagents. Sodium butyrate (NaBu), chlorpromazine, and EpCAM ELISA kit were purchased from Sigma-Aldrich Inc. (U.S.A.). ManNAz, GalNAz, lipofectamine RNAiMAX (Lipo), Alexa Fluor 647 DIBO alkyne, and FITC-conjugated EpCAM mouse antihuman mAb (clone VU-1D9) (EpCAM anti) were obtained from Life Technologies (U.S.A.). Exonuclease III and 1× NE buffer were obtained from New England Biolabs (U.K.). MCF-7 and Ramos cells were from KeyGen Biotech. Co. Ltd. (Nanjing, China). Tris−EDTA (TE) buffer containing 10 mM Tris−HCl and 1 mM EDTA (TE, pH 8.0) was used as DNA stock solution. Phosphate buffer saline (PBS, pH 7.4) contained 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4, and 1.41 mM KH_2PO_4. To reduce the nonspecific binding of aptamer to cells, yeast tRNA (0.1 mg/mL, Sigma) was added in PBS containing 5 mM $MgCl_2$, which was used as the washing buffer. All other reagents were of analytical grade. All aqueous solutions were prepared using ultrapure water (≥18 MΩ, Milli-Q, Millipore).

All DNA probes with following sequences were customized from Sangon Biological Engineering Technology & Co. Ltd. (Shanghai, China): protein probe (PP), 5′-AAAAAAAAAA-AAAAAAAAAAAAAAAAAAAA/iSpC18/CACTACAGAGGT-TGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCT-G-3′; glycan probe (GP), 5′-DIBO/iSpC18/TTTTTTTTT-TTTTTTTTTTTT/iBHQdT//iFAMdT/TTTTTTT-3′; fluorescein protein probe (FPP), 5′-Cy5-AAAAAAAAAAAAAAAAAAAAAAAAAAAAA/iSpC18/CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGG-GGGGTTGGCCTG-3′; fluorescein glycan probe (FGP), 5′-DIBO/iSpC18/TTTTTTTTTTTTTTTTTTTTT/iFA-MdT/TTTTTTT-3′.

The underlined sequence in PP and FPP was the aptamer of epithelial cell adhesion molecule (EpCAM).[23] A random sequence probe was obtained as blank control by replacing the aptamer in FPP with a random DNA sequence. Human EpCAM siRNA,[19] 5′-CUACAAGCUGGCCGUAAACdTdT-3′, and 5′-GUUUACGGCCAGCUUGUAGdTdT-3′ and control siRNA, 5′-UUCUCCGAACGUGUCACGUTdTd-3′, and 5′-ACGUGACACGUUCGGAGAATdTdT-3′, were purchased from Shanghai GenePharma Co. Ltd. (Shanghai, China).

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Apparatus. The fluorescence spectra were recorded on an F-7000 fluorescence spectrophotometer (Hitachi, Japan). Flow cytometric analysis was performed on a Coulter FC-500 flow cytometer (Beckman-Coulter). The fluorescence cell imaging was performed on a TCS SP5 laser scanning confocal microscope (Leica, Germany). EpCAM ELISA was performed on a Varioskan Flash full wavelength scanning multifunction reading meter (ThermoFisher Scientific, U.S.A.).

Cell Culture and Metabolic Labeling of Cell Surface Glycans. MCF-7 and Ramos cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (FCS, Sigma), penicillin (100 μg/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere containing 5% CO_2. The cell numbers were determined using a Petroff-Hausser cell counter (U.S.A.).

The 50 mM metabolic labeling reagents, ManNAz (for labeling cell surface sialic acid) and GalNAz (for labeling cell surface O-linked glycans), were prepared in DMSO as the stock solutions. MCF-7 cells were incubated for 48 h in culture medium containing one of the metabolic labeling reagents at 50 μM. The cells were then trypsinized and washed with PBS for three times before measurement.

Flow Cytometric Analysis of Cell Surface EpCAM and Glycan. To confirm the existence of EpCAM on MCF-7 cell surface, an EpCAM negative cell line Ramos was used as control. MCF-7 and Ramos cells with a concentration of 1 × 10^6 cells/mL were incubated with 20% FITC-conjugated EpCAM mouse antihuman mAb in PBS or 1 μM FPP in binding buffer, respectively. After 30 min of incubation at 37 °C, the cells were washed with PBS or washing buffer twice before flow cytometric test. For investigation of the dynamic change of cell surface EpCAM during NaBu treatment, MCF-7 cells were incubated with 1 mM NaBu for 1−7 days and subsequently analyzed with the same procedure. The Sia change on whole cell surface was analyzed by incubating NaBu-treated and then ManNAz metabolically labeled MCF-7 cells with 1 μM FGP for 30 min at 37 °C. The cells were washed with PBS twice before flow cytometric detection.

Quantification of EpCAM-Specific Sia on the Cell Surface. The metabolically labeled MCF-7 cells were first incubated with 1 μM PP in binding buffer containing 5 μg/mL chlorpromazine at 37 °C for 30 min. After the cells were washed twice with washing buffer, fixed with 75% ethanol for 15 min, and incubated with 1 μM GP in PBS at 37 °C for 30 min, they were washed twice with PBS and incubated with 100 U/mL Exo III in the mixture of 1× NE buffer, TE buffer, 100 mM NaCl, and 10 mM $MgCl_2$ at 37 °C. After incubation for 3 h, the obtained mixture was subjected to fluorescence detection for quantification of EpCAM-specific Sia on cell surface.

RNA Interference and ELISA Analysis of EpCAM on MCF-7 Cells. MCF-7 cells were transfected with 10 μM siRNA in Lipo for RNA interference and 50 μM ManNAz for metabolic labeling using serum-free medium at 37 °C for 48 h according to the manufacturer's protocol.

After different concentrations of MCF-7 cells were washed with cold PBS by centrifugation at 1000 rpm twice, they were resuspended in cell lysis solution (PBS pH 7.4, 0.5% Tween-20, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 min with frequent vortexing. The mixtures were centrifuged at 13 000 rpm for 10 min. The obtained supernatants were subjected to ELISA analysis using EpCAM ELISA kit by the manufacturer's protocol. From the standard curve (Figure S7) and the absorbance of cell lysis solution, the average amount of EpCAM on each MCF-7 cell was determined to be 0.00946 pg, which produced the average number of EpCAM on each cell to be 1.6 × 10^5 from the EpCAM mass of 34 932 Da.

RESULTS AND DISCUSSION

In Vitro Release of Fluorescein. The Exo III-aided release of fluorescein was first demonstrated in vitro (Figure S1). The designed PP and GP and their mixture did not show fluorescent signal, neither did Exo III-treated PP or GP. However, after the mixture PP and GP was incubated and then treated with Exo III, obvious fluorescent signal of fluorescein was observed, indicating the successful release of fluorescein from GP in the presence of Exo III.

Specificity of Protein Recognition. To demonstrate the specificity of aptamer to EpCAM on the cell surface, a fluorescent protein probe (FPP) was prepared by labeling Cy5 at the 5′ end of PP, and a random sequence probe was obtained as blank control by replacing the aptamer sequence with a random DNA sequence. After the MCF-7 cells were treated with FITC-conjugated EpCAM antibody, FPP, and random sequence probe, respectively, flow cytometric analysis exhibited strong binding of both the antibody and FPP, and no binding of random sequence probe to MCF-7 cells was observed. Moreover, these treatments did not show any response on EpCAM-negative Ramos cells (Figure S2). These results suggested the specific binding of aptamer in PP to EpCAM on MCF-7 cell surface.

Selectivity of Protein-Specific Glycan. The selectivity of the designed glycosylation information liberation from target protein was verified using fluorescence spectroscopy (Figure 2).

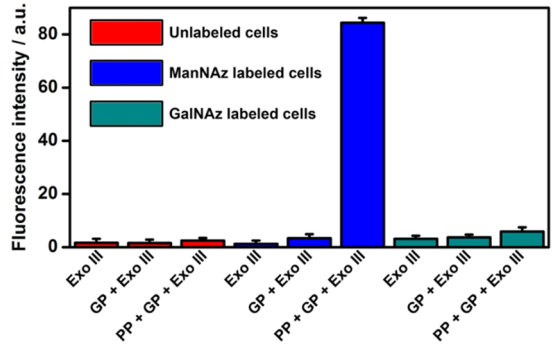

Figure 2. Fluorescence intensity of unlabeled, ManNAz-labeled, or GalNAz-labeled 5 × 10^6 cells/mL MCF-7 cells solution after being incubated with 100 U/mL Exo III for 3 h, 10 μM GP for 30 min, and 100 U/mL Exo III for 3 h, 10 μM PP for 30 min followed with 10 μM GP for 30 min, and 100 U/mL Exo III for 3 h at 37 °C.

After MCF-7 cells were metabolically labeled with GalNAz or ManNAz and then Alexa Fluor 647 DIBO alkyne, they showed strong fluorescent signal on the cell surface (Figure S3), indicating the existence of both Sia and OLG on MCF-7 cell surface. Upon stepwise incubation of the labeled MCF-7 cells with PP, GP, and Exo III, only the solution of ManNAz metabolically labeled cells showed liberated fluorescent signal (Figure 2). Considering the fact that EpCAM has no O-linked glycosylation site[20] and PP can selectively bind cell surface EpCAM, negligible fluorescence liberation of GalNAz metabolically labeled cells indicated the GP bound on OLG that located beyond the efficient distance hybridizable with PP could not hybridize with PP bound on EpCAM to liberate the

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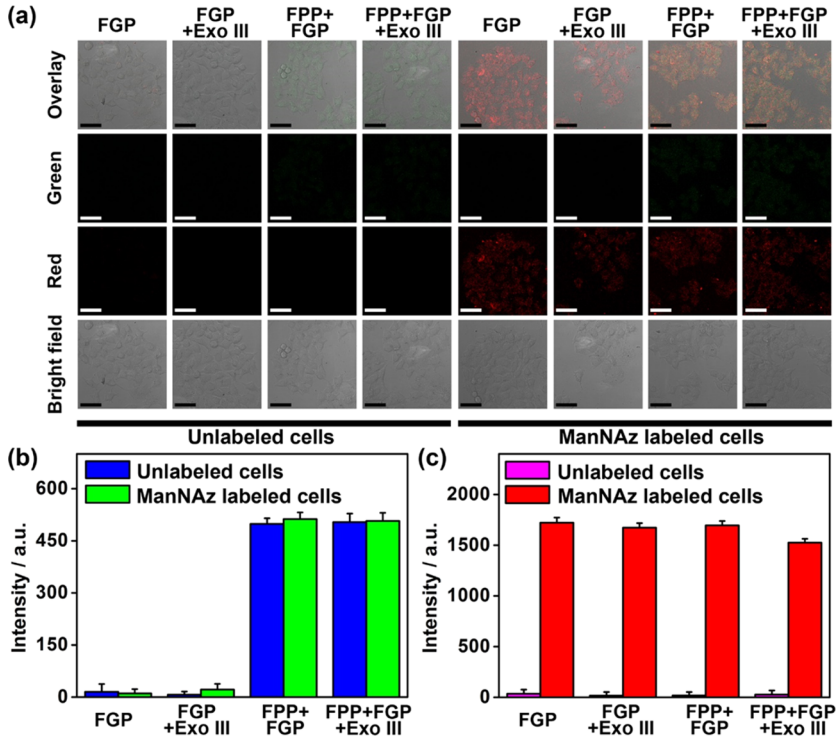

Figure 3. (a) Confocal dual-color images of unlabeled and ManNAz-labeled MCF-7 cells after being incubated with 10 μM FGP for 30 min, 10 μM FGP for 30 min, and then 100 U/mL Exo III for 3 h, 10 μM FPP for 30 min, and then 10 μM FGP for 30 min, or 10 μM FPP for 30 min followed with 10 μM FGP for 30 min, and then 100 U/mL Exo III for 3 h at 37 °C. The green and red channels represent fluorescence of Cy5 of FPP and FAM of FGP, respectively. Scale bar: 50 μm. Fluorescence intensity of green (b) and red (c) channels obtained from panel a.

fluorescence signal. Thus, the designed spacer can exclude the hybridization possibility of PP with the GP bound on non-protein-specific glycan. Only the GP bound on EpCAM-specific Sia could release the fluorescent molecule via the designed Exo III-aided recycling hybridization. After EpCAM was knocked down from the cell surface with an RNAi experiment (Figure S4, parts a and b), the MCF-7 cells treated with PP, GP, and Exo III did not show liberated fluorescence (Figure S4c), further indicating the selectivity.

Dual-Color Confocal Fluorescence Imaging. The stability of the PP−EpCAM complex and Exo III-aided recycling hybridization of PP and GP on the protein-specific glycan was first observed with dual-color confocal fluorescence imaging using FPP and fluorescein glycan probe (FGP) (Figure 3a). The FGP was obtained for fluorescence labeling of glycan by removing the quencher BHQ from the GP. After incubation with FGP, the ManNAz-labeled cells displayed obvious fluorescent signal of the corresponding glycan while the unlabeled cells did not (Figure 3, parts a and c), indicating the specificity of glycan binding. After being incubated with FPP, all cells showed the fluorescence of Cy5 in the FPP with a green channel (Figure 3b), indicating the recognition of PP to EpCAM on MCF-7 cells. Upon the further incubation with FGP or/and Exo III, the fluorescent signal of Cy5 kept at a constant intensity (Figure 3b), which demonstrated the good stability of the formed PP−EpCAM complex. The incubation with FGP also led to the occurrence of FAM fluorescence, which decreased for about 10% upon incubation with Exo III (Figure 3c), indicating the binding of FGP to cell surface glycan and the cleavage of FAM from the hybridized FGP and recycling hybridization of FPP with FGP bound on the protein-specific glycan.

Quantification of Protein-Specific Glycan. Prior to the homogeneous quantification of EpCAM-specific Sia with the released FAM, the metabolic labeling time with ManNAz was optimized to be 48 h using confocal fluorescence imaging with Alexa Fluor 647 DIBO alkyne (Figure S5), and the incubation times of PP, GP, and Exo III were optimized to be 30 min, 30 min, and 3 h, respectively (Figure S6). When ManNAz-labeled cells were incubated with PP, chlorpromazine was used as endocytosis inhibitor to prevent the endocytosis of PP. During the continuous incubation processes, the cells were fixed to keep their structural integrity.

In homogeneous solution the fluorescent intensity (I) of fluorescein was proportional to its concentration (c_{FAM}) (Figure 4, parts a and b):

$$I = k_1 c_{FAM} + b_1 \quad (1)$$

The I value of fluorescein released from the cell surface during the Exo III-aided recycling hybridization was proportional to cell concentration (c_{cell}) in the range from 1×10^6 to 5×10^6 cells mL^{-1} (Figure 4, parts c and d):

$$I = k_2 c_{cell} + b_2 \quad (2)$$

The amount of fluorescein released from each cell was defined as a_{FAM}; then eq 1 could be transformed to

$$I = k_1 a_{FAM} c_{cell} + b_1 \quad (3)$$

Theoretically, both b_1 and b_2 should be 0. Thus, the following eq 4 could be obtained from eqs 2 and 3:

$$a_{FAM} = k_2/k_1 \quad (4)$$

From the slopes, the a_{FAM} could be calculated to be 3.8×10^6 per cell, which could be regarded as the amount of EpCAM-

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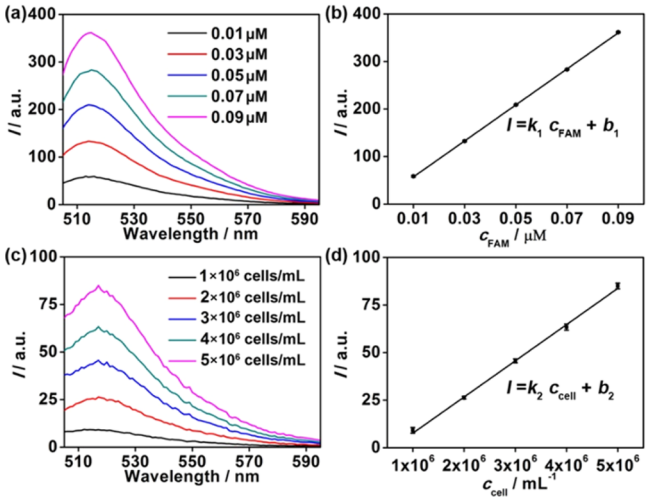

Figure 4. (a) Fluorescence spectra of FAM at different concentrations and (b) plot of fluorescent intensity vs FAM concentration. (c) Fluorescence spectra of metabolically labeled MCF-7 cells after continuous incubation with PP, GP, and Exo III and (d) plot of fluorescent intensity vs cell concentration.

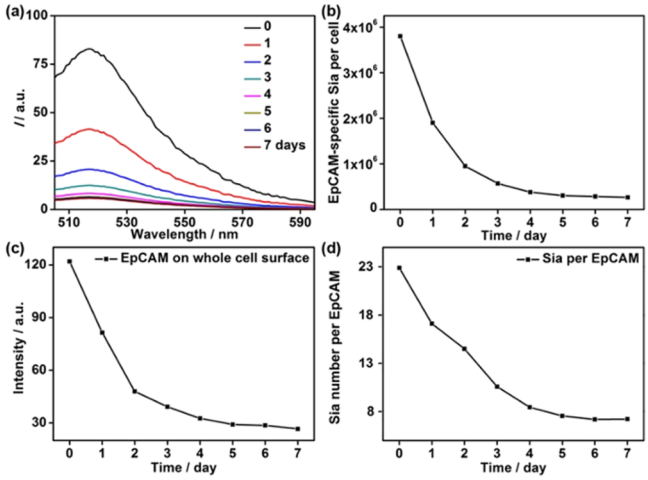

Figure 5. (a) Fluorescence spectra of 5×10^6 cells/mL MCF-7 cells subjected to EpCAM-specific Sia quantification after being treated with 1 mM NaBu for different days and plots of (b) EpCAM-specific Sia on each cell, (c) mean fluorescent intensity by flow cytometric analysis with FITC-conjugated EpCAM antibody, and (d) Sia number on each EpCAM vs NaBu treatment time.

specific Sia on each cell when the incompleteness of metabolic labeling was ignored. It was about 1/10 of Sia expression on the whole MCF-7 cell surface reported by the previous work,[24] which was in good agreement with the decrease of FAM fluorescence of FGP bound on whole cell surface (Figure 3c). This result indicated the validity of the proposed protocol.

Glycosylation Variation of Protein-Specific Glycan. The proposed liberation strategy of protein-specific glycan signal could be used to quantify glycosylation level and its change of specific protein. The average amount of EpCAM expressed on each cell was quantified to be 1.6×10^5 by the ELISA method (Figure S7). So Sia expressed on each EpCAM of an MCF-7 cell could be calculated as 23. After MCF-7 cells were treated with 1 mM NaBu, which can induce the change of protein and glycan expression level on the cell surface,[25−27] for different times, the cells were labeled with ManNAz and subjected to quantification of EpCAM-specific Sia. The expression of EpCAM-specific Sia on each cell decreased obviously with the increasing treatment time from 1 to 7 days (Figure 5, parts a and b). Flow cytometric analysis also showed the down regulation of EpCAM expression on whole cell surface (Figure 5c). When coupled with the ELISA analysis of EpCAM expression during NaBu treatment, the Sia expressed on each EpCAM decreased from 23 to 7 (Figure 5d). Considering the facts that the common structure of N-linked glycans contains 2−4 terminal Sia groups,[28−30] EpCAM contains three potential N-linked glycosylation sites, and two of them can be glycosylated,[20] the stable level of 7 for Sia expressed on each EpCAM after NaBu treatment was reasonable. The expression level of 23 for EpCAM-specific Sia on MCF-7 cell without any treatment indicated the aberrant hypersialylation of EpCAM on cancer cells.

Different from the EpCAM-specific Sia detected with the proposed protocol, flow cytometric analysis with FGP showed the increased expression of Sia on whole cell surface under NaBu treatment (Figure S8). This might be attributed to the up-regulated glycosylation of other glycoproteins.[31,32] Thus, the precise quantification for protein-specific glycosylation level is necessary in uncovering glycosylation-related biological processes.

CONCLUSION

In conclusion, this work designs two probes and an Exo III-aided recycling hybridization strategy for liberation of protein-specific glycosylation information and in situ homogeneous quantification of protein-specific glycan on the cell surface. These probes can selectively bind to target protein and specific glycan, respectively. Moreover the complex of target protein and the protein probe shows good stability. In an efficient hybridizable distance, the hybridization of protein probe with glycan probe forms a duplex DNA, which leads to the cleavage of the hybridized glycan probe by Exo III to release the fluorescein and the further hybridization of the left protein probe with the other adjacent glycan probe to achieve the recycling hybridization and the release of the labeled fluorescein bound to all protein-specific glycan. Due to the controllable length of the designed spacer in the probes, the liberation of protein-specific glycan shows good specificity. Thus, the amount of protein-specific glycan can be conveniently quantified with the liberated signal through homogeneous detection. This strategy has been used to in situ quantify the target protein EpCAM-specific sialic acid on MCF-7 cell surface and monitor the variation of sialylation level of cell surface EpCAM during drug treatment, indicating a promising quantification tool for research of protein glycosylation in biological processes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b04883.

Fluorescence spectra of probes, flow cytometric data and fluorescence images of cells, RNAi and ELISA analysis, optimization of conditions (PDF)

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Author Contributions

[§]Y.C. and L.D. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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