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Detection of Glycans

A Bioorthogonal Raman Reporter Strategy for SERS Detection of Glycans on Live Cells**

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Glycans on cell surfaces participate in cell–cell communication and host–pathogen interactions.[1] Moreover, they change in structure and expression in the course of many physiological processes including development and disease (e.g., cancer) progression. Detecting or visualizing glycans on live cells yet remains an ongoing challenge because of the nongenetically encoded nature of glycosylation. To this end, a two-step chemical reporter strategy has proven to be a powerful tool.[2] First, an unnatural monosaccharide analog bearing a bioorthogonal functional group or a chemical reporter (e.g., azide or alkyne) is metabolically incorporated into target glycans by the cellular biosynthetic machinery. In a second step, the chemical reporter is covalently conjugated with a fluorescent probe bearing a complementary functionality using a bioorthogonal chemical reaction (Scheme 1 a). This chemical reporter strategy hinges on the utility of chemical reactions that meet the stringent requirements of "bioorthogonality". The bioorthogonal chemical groups must be nonnative and small enough to be tolerated by the enzymes of the cell. Furthermore, the bioorthogonal reaction step requires two participating groups to be mutually reactive while remaining inert to the surrounding molecules in the physiological environment. Only a handful of chemical reactions satisfy such requisite requirements that are available for visualization of glycans including Staudinger ligation,[3] Cu^{I}-catalyzed azide–alkyne cycloaddition (CuAAC),[4] strain-promoted azide–alkyne cycloaddition,[5] and cyclopropene–tetrazene cycloaddition.[6] On the other hand, improving the reaction kinetics and biocompatibility of the current reactions is needed for studies performed in complex biological systems such as inside living animals. Indeed, development of new bioorthogonal reactions is currently a very active field of research.[7]

Alternatively, we sought to directly detect unnatural sugars on cell surfaces and therefore obviate the need of the second-step bioorthogonal labeling (Scheme 1 b). To do so, the unnatural sugars must be modified with a reporter that can be directly detected without further chemical derivatization. The substrate promiscuity of the glycan biosynthetic pathways is restricted to certain modifications depending on the enzymes involved. The bulkiest group that has been metabolically incorporated into cell surface glycans is the phenyl azide appended to sialic acid,[8] which is probably the most versatile monosaccharide for accommodating substituents.[9] Fluorescent dyes are generally too large in size to be directly installed on monosaccharides for metabolic incorporation. To overcome this limitation, we alternated means of detection and turned to exploit Raman spectroscopy and microscopy.

Raman spectroscopy relies on Raman scattering to detect specific vibrational signals of molecules. In principle, any chemical group can be a Raman tag, so that the size criterion of the substituents on monosaccharides can be readily fulfilled. Raman signals of natural sugars, mainly composed of C–H and C=O vibrational stretches, are overwhelmed by the signals generated by the same functional groups found in massive lipids and proteins in cells. Therefore, the use of unnatural sugars modified with a reporter, the Raman scattering of which is "bioorthogonal", is desired. The bioorthogonality in this case is defined as that the Raman reporter possesses a vibration in the Raman-silent region of a cell (approximately 1800 to 2800 cm^{-1}), where the Raman signals from cellular components are negligible.

Interestingly, the alkyne and azide—two chemically bioorthogonal groups that are most widely used—are also Raman spectroscopically bioorthogonal with Raman scattering at about 2100 cm^{-1}. Recently, the Sodeoka group demonstrated the use of alkyne as a bioorthogonal Raman reporter for imaging nuclear DNA using the EdU (5-ethynyl-2′-deoxyuridine) probe[10] and imaging intracellular alkyne-tagged coenzyme Q (CoQ).[11] However, detection of glycans

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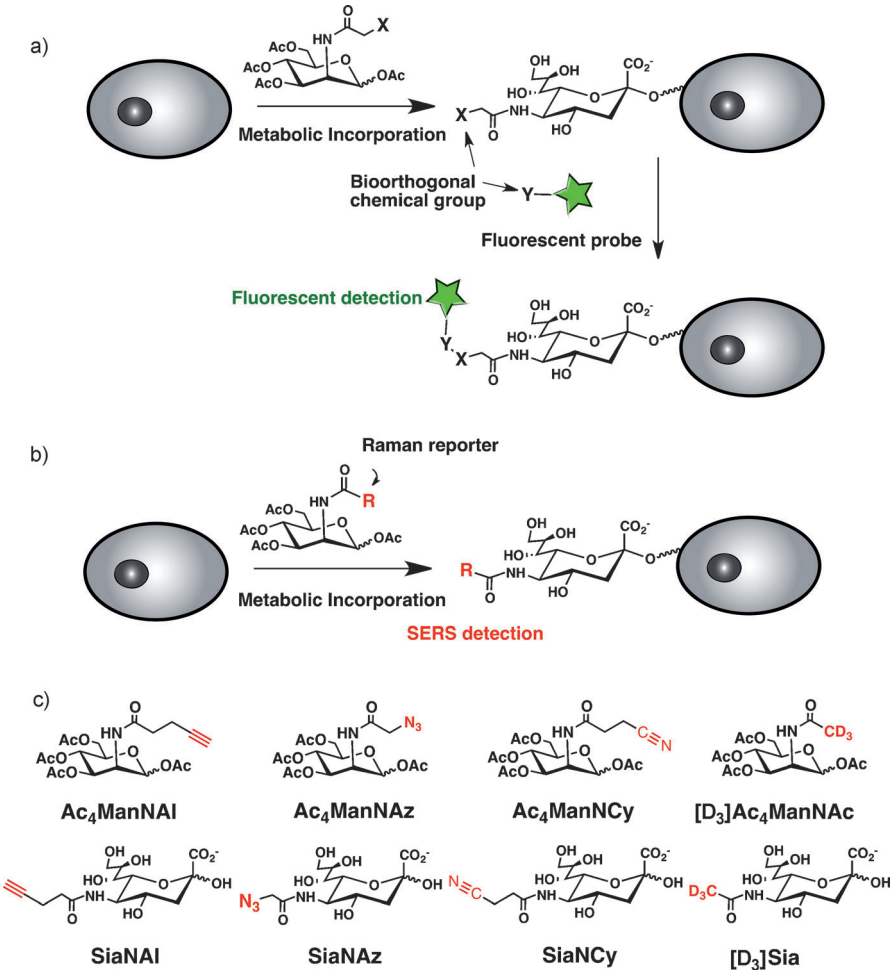

Scheme 1. a) The two-step chemical reporter strategy for visualizing sialylated glycans on cell surfaces. First, a ManNAc analog containing a biorothogonal functional group is metabolically converted to the corresponding sialic acid analog and incorporated into sialylated glycans. Second, a fluorescent probe containing a complementary bioorthogonal group is introduced using a bioorthogonal reaction. b) The bioorthogonal Raman reporter strategy. A ManNAc analog installed with a bioorthogonal Raman reporter is metabolically incorporated into silaylated glycans on cell surfaces, which is directly detected by SERS. c) Monosaccharide analogs containing a Raman reporter used in this study. The alkyne and azide can serve as both chemical reporters and Raman reporters. Ac_4ManNCy and $[D_3]Ac_4$ManNAc contain two new Raman reporters, the nitrile group and the C–D bond, respectively, which are not amenable to bioorthogonal reactions. $[D_3]Ac_4$ManNAc is designed with three C–D bonds as the Raman reporter, which induces no structural modification but an isotopic substitution, and hence minimal functional perturbation.

on live cells using the bioorthogonal Raman reporter strategy has yet to be achieved.

The major roadblock for cellular Raman spectroscopy and microscopy has been the intrinsic weakness of the Raman signal and hence the poor sensitivity. In the case of DNA imaging,[10] EdU is incorporated into DNA as a thymidine analog during DNA replication and accumulated in cell nucleus with high local concentration, and therefore it can be detected by intrinsic Raman microscopy. However, glycans mainly reside on the cell surface as a two-dimensional layer, so the local concentration of sugar molecules inside a focal volume is improbable to reach the detection limit of intrinsic Raman spectroscopy. To overcome this difficulty, we turned to an enhancing technique, surface-enhance Raman scattering (SERS), which improves the sensitivity up to single-molecule level.[12] Herein, we demonstrate the SERS detection of sialylated glycans metabolically incorporated with a bioorthogonal Raman reporter on live cells (Scheme 1b).

We first evaluated the alkyne as a bioorhtogonal Raman reporter for SERS detection of sialylated glycans on live cells. We exploited peracetylated *N*-(4-pentynoyl)mannosamine (Ac_4ManNAl), which is converted to the corresponding sialic acids (SiaNAl) upon feeding to cells and incorporated into cell surface glycans (Scheme 1c). Before performing SERS experiments, we characterized the intrinsic Raman spectra of Ac_4ManNAl and SiaNAl, which showed an alkyne peak at 2120 cm^{-1} for Ac_4ManNAl and 2113 cm^{-1} for SiaNAl (see Figure S1 in the Supporting Information). For SERS detection, we synthesized gold plasmonic nanoparticles (AuNPs) with the diameter of about 120 nm as the Raman signal amplifier (see Figure S2 in the Supporting Information). The enhancement drops precipitously as the distance between the AuNP surface and the Raman tag increases.[13] The AuNPs were hence functionalized with 4-mercaptophenylboronic acid (MPBA-AuNPs) to facilitate their localization in close proximity to the sialylated glycans. The phenylboronic acid moiety preferentially binds to sialic acids,[14] and therefore ensures the efficient amplification of the Raman signal (see Scheme S1 in the Supporting Information). The SERS spectra of the SiaNAl and the natural sialic acid were then obtained on the MPBA-AuNP substrate. The alkyne peak of SiaNAl in SERS spectra was observed at about 2121 cm^{-1}, and there was no peak observed within the silent region for natural sialic acid (Figure 1a).

To detect the sialylated glycans on cell surfaces by SERS, HeLa cells were treated with Ac_4ManNAl to incorporate the corresponding SiaNAl into cell surface glycans. The presence of alkyne on the cell surface was confirmed by conjugation with a fluorescent dye, followed by detection using flow cytometry as previously described (data not shown).[15] The Ac_4ManNAl-treated cells were then incubated with MPBA-AuNPs as the SERS signal amplifier. The dark-field microscopy confirmed the presence of MPBA-AuNPs on the cell surface, shown as bright spots resulting from the plasmon light scattering of Au nanoparticles (Figure 1b).[16] The uneven

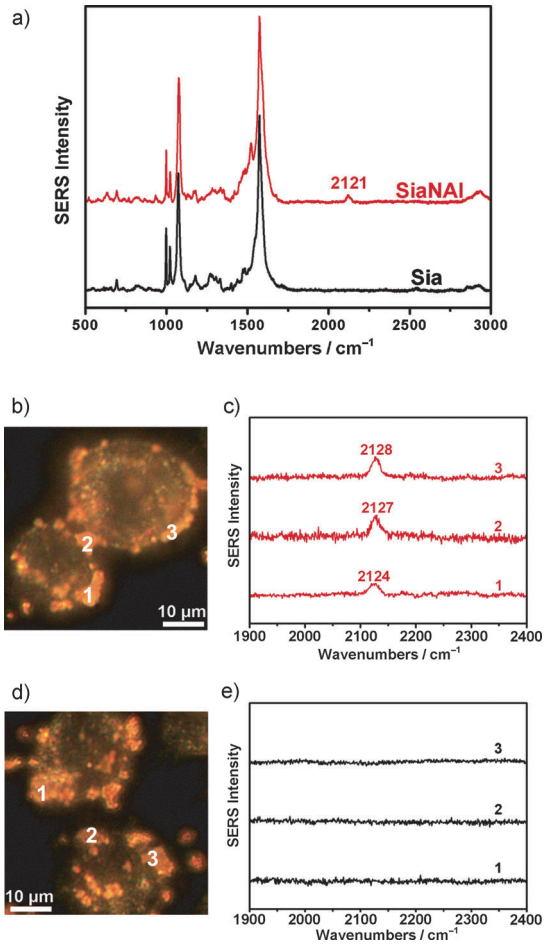

Figure 1. SERS detection of SiaNAl on cell surfaces. a) SERS spectra of SiaNAl and natural sialic acid (Sia) obtained in PBS solution. b) Dark-field image of HeLa cells treated with Ac_4ManNAl, followed by incubation with MPBA-AuNPs. The scattering bright spots indicate the gold nanoparticles on the cell surfaces. c) Representative SERS spectra of HeLa cells treated with Ac_4ManNAl, which were taken at three locations on the cell surfaces as indicated in the dark-field image on the left. d and e) Dark-field image and SERS spectra of control cells treated with Ac_4ManNAc. The SERS data shown are representative of at least 100 measurments.

distribution of AuNPs was observed at certain locations on cell surfaces, which probably resulted from the clustering of AuNPs. In our detection method, the SERS signal comes diretly from the alkynyl sugars on the cell surface, so the distribution of AuNPs does not affect the specificity of the SERS detection. The SERS spectra were then taken at various cell surface locations (more than 100 replicates), and almost all of them showed a SERS peak at about 2126 cm^{-1} corresponding to the alkynyl group of SiaNAl on the cell surfaces (Figure 1c and see Figure S3 in the Supporting Information). When SERS spectra were taken on control cells treated with Ac_4ManNAc, no alkyne signal was detected in all replicate experiments (Figure 1d and e). The SERS detection of SiaNAl-incorporated glycans on the cell surface can also be applied to other cell lines such as CHO cells (see Figure S4). These results collectively demonstrate that SERS can be used to directly detect alkyne-incorporated sialylated glycans on cell surfaces. Importantly, the Raman reporter, an alkynyl group, is installed on sialic acids directly, so the SERS signals at about 2126 cm^{-1} on the cell surface exclusively result from the alkyne-incorporated sialylated glycans. This feature endows our method with signal specificity. The nonspecific signal does not exist in the control cells treated with Ac_4ManNAc, independent of the presence of AuNPs.

One of the intriguing characteristics of SERS is the great sensitivity.[17] Moreover, SERS detection of alkynes does not have cellular background, an issue that often compromises sensitivity in fluorescent detection. Taken together, we envisioned that SERS would be well-suited for detecting low abundant biomolecules on cell surfaces. To evaluate the sensitivity of SERS detection of cell surface glycans, we treated CHO cells with Ac_4ManNAl at a series of concentrations ranging from 50 to 0.1 μM. The SERS signal of SiaNAl on the cell suface can be easily detected at all concentrations (Figure 2). By comparison, flow cytometry detection of

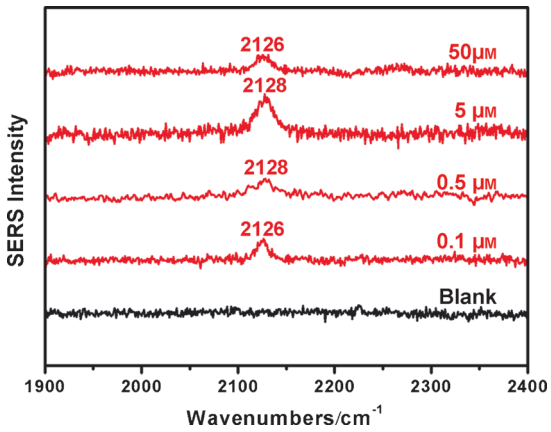

Figure 2. Evaluation of the sensitivity of SERS detection of SiaNAl on cell surfaces. CHO cells were treated with Ac_4ManNAl at concentrations indicated and analyzed by SERS.

SiaNAl-incorporated glycans on cell surfaces that are chemically conjugated with a fluorescent probe (Scheme 1a) usually requires treating cells with Ac_4ManNAl above the 0.5 μM level (see Figure S5). These results indicate that the sensitivity of SERS detection of cell surface glycans is comparable to or even better than that of fluorescent detection. However, it should be noted that the SERS enhancement or intensity is strongly dependent on the distance between SiaNAl and AuNPs, which makes quantifying the SERS signal a challenging task.

Prompted by the results on SERS detection of the alkyne-incorported glycans, we sought to evaluate whether azide can serve as another bioorthogonal Raman reporter. Although it was shown that the relative Raman intensity of azide is only about 1/7 of the intensity of alkyne in hexanoic acid analogs,[11] we expected SERS detection of azido sialic acid (SiaNAz) on cell surfaces would be feasible. Simiarly, we first charaterized the intrinsic Raman spectroscopy and SERS of azido sugars. Interestingly, the vibrational frequency of azide in peracetylated *N*-azidoacytelmannosamine (Ac_4ManNAz)

and SiaNAz are 2112 and 2130 cm^{-1}, respectively, exhibiting a shift of about 20 cm^{-1} (see Figure S6 in Supporting Information). The azide peak of SiaNAz in SERS was observed at 2129 cm^{-1}, also in the Raman-silent region (Figure 3a). HeLa cells were then treated with 50 μM Ac_4ManNAz. As expected, the cell surface SiaNAz was

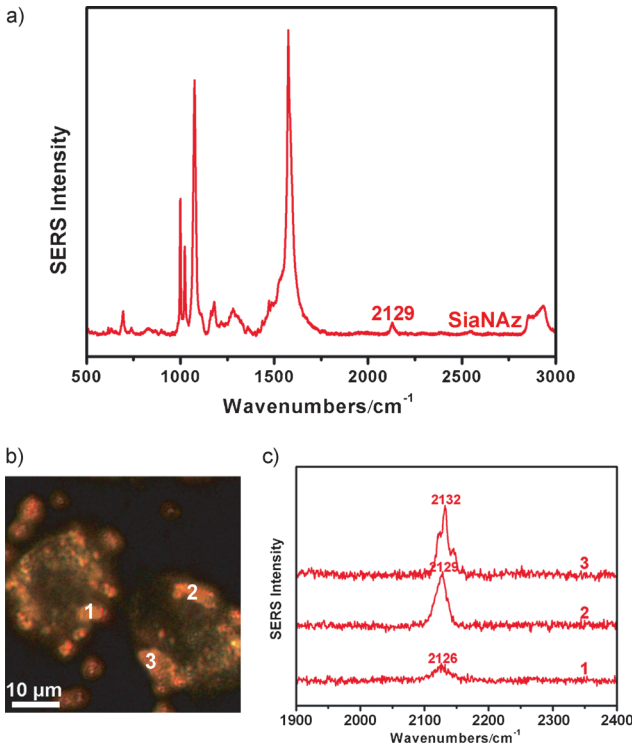

Figure 3. SERS detection of SiaNAz-incorporated glycans on HeLa cells treated with Ac_4ManNAz. a) SERS spectrum of SiaNAz obtained in PBS solution. b) Dark-field image of HeLa cells treated with Ac_4ManNAz, followed by incubation with MPBA-AuNPs. c) Representative SERS spectra taken at three locations on cell surfaces as indicated in the dark-field image on the left. The SERS data shown are representative of at least 100 measurments.

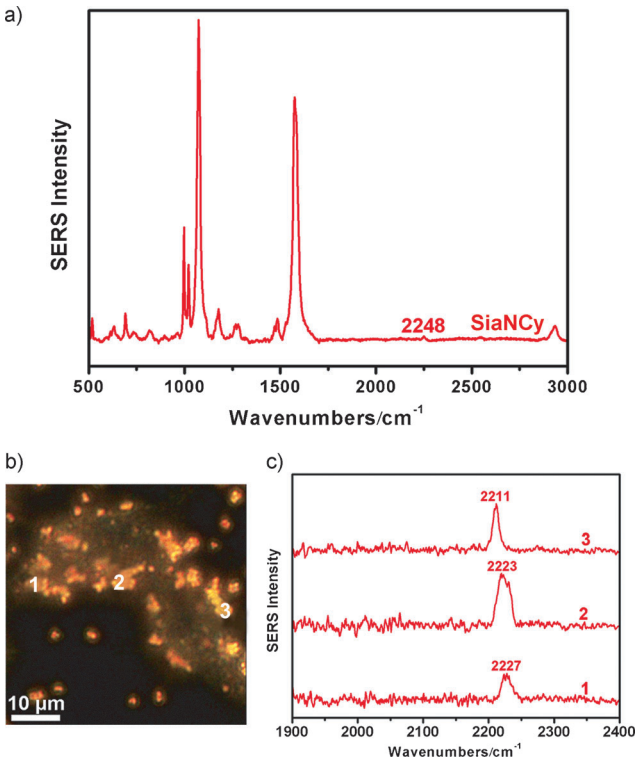

Figure 4. Development of the nitrile group as a bioorthogonal Raman reporter that is not amenable to bioorthogonal reaction. a) SERS spectrum of SiaNCy obtained in PBS solution. b) Dark-field image of HeLa cells treated with Ac_4ManNCy, followed by incubation with MPBA-AuNPs. c) Representative SERS spectra taken at three locations on cell surfaces as indicated in the dark-field image on the left. The SERS data shown are representative of at least 100 measurments.

readily detected by SERS (Figure 3b and c, and see Figure S7 in the Supporting Information). Similar results were obtained in CHO cells (data not shown).

Having established the SERS detection of alkyne- and azide-labeled glycans, we sought to expand the scope of the bioorthogonal Raman reporter. By circumventing the constraints of bioorthogonal chemistry, our methodology enables the almost unlimited choices of Raman reporter. To demostrate this feature, we developed two new functional groups as Raman reporter, the nitrile group and the carbon–deuterium bond (C–D), which are not amenable to bioorthogonal reactions. We chose nitrile because its size is as small as that of alkyne, which should ensure its metabolic incorporation. The Raman intensity of the nitrile is about half that of the alkyne.[11] We therefore synthesized peracetylated *N*-(3-cyanopropanoyl)mannosamine (Ac_4ManNCy) and the corresponding cyano sialic acid (SiaNCy; Scheme 1c and see the Supporting Information for the synthetic procedure). The intrinsic Raman spectroscopy showed the vibrational frequency of nitrile at 2251 cm^{-1} for Ac_4ManNCy and 2252 cm^{-1} for SiaNCy (see Figure S8 in the Supporting Information). The nitrile peak of SiaNCy in SERS was also observed in the Raman-silent region at 2248 cm^{-1} (Figure 4a). We then treated HeLa cells with 50 μM Ac_4ManNCy, and detected the nitrile-incorporated sialylated glycans on the cell surface using SERS. SiaNCy on the cell surfaces clearly exhibited a SERS peak at about 2220 cm^{-1} (Figure 4b and c). These results demostrated that the cyano group can serve as a bioorthogonal Raman reporter for SERS detection.

Although the azide, alkyne, and nitrile groups are relatively small in size and chemically inert to the biological milieu, they may still cause structural perturbations that interfere with the biological functions of sialic acids such as receptor binding.[9] For example, substitution of an azido group at the C-9 position of sialic acid abolished the binding of the influenza C virus.[18] The fact that Raman reporters can be designed as small as a single chemical bond prompted us to develop new Raman reporter-containing ManNAc analogs that cause minimal perturbation to the biological functions of sialylated glycans. We envisaged that deuterated monosaccharides, that is, replacing C–H bonds with C–D bonds, would be ideal for this purpose. The deuteration shifts the Raman vibrational frequency from about 2890 cm^{-1} for C–H to about

2117 cm^{-1} for C–D, falling right into the Raman-silent region (see Figure S9 in the Supporting Information). Furthermore, the isotopic substitution with deuterium does not interfere with the chemical and biological properties of its unlabeled counterpart, as exemplified by its wide use in tracing the metabolic pathways. We therefore synthesized peracetylated *N*-trideutero-acetylmannosamine ($[D_3]Ac_4ManNAc$) and the corresponding deuterated sialic acid ($[D_3]Sia$; Scheme 1c and see the Supporting Information for the synthetic procedure). The Raman spectra showed that $[D_3]Sia$ possesses a strong peak within the Raman-silent region of a cell (see Figure S9 in the Supporting Information). The C–D peak of $[D_3]Sia$ in the SERS spectrum was observed at about 2122 cm^{-1} (Figure 5a).

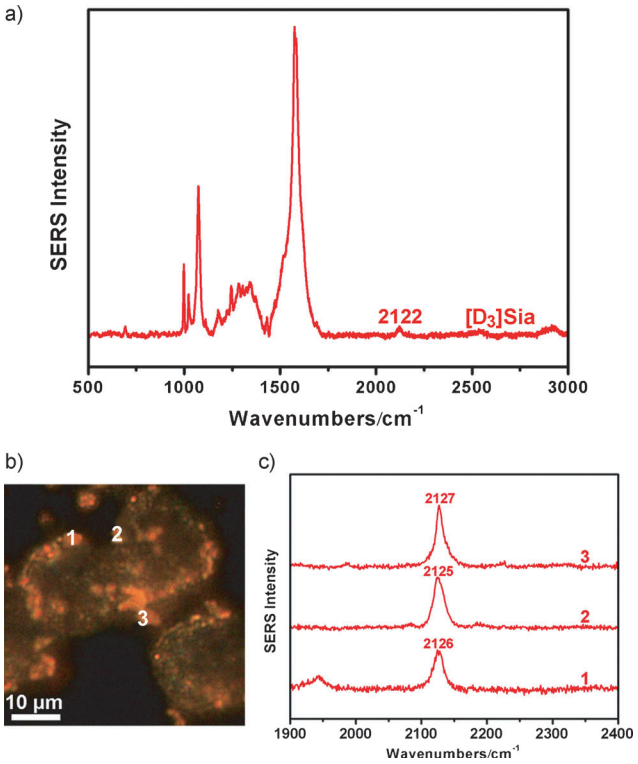

Figure 5. Probing cell surface glycans using C–D as a bioorthogonal Raman reporter. a) SERS spectrum of $[D_3]Sia$ obtained in PBS solution. b) Dark-field image of HeLa cells treated with $[D_3]Ac_4ManNAc$, followed by incubation with MPBA-AuNPs. c) Representative SERS spectra taken at three locations on cell surfaces as indicated in the dark-field image on the left. The SERS data shown are representative of at least 100 measurments.

We then treated HeLa cells with $[D_3]Ac_4ManNAc$ and the treated cells were subjected to SERS analysis. As expected, we observed a SERS peak at about 2125 cm^{-1}, corresponding to $[D_3]Sia$ on the cell surfaces (Figure 5b and c). There results demonstrated that $[D_3]Ac_4ManNAc$ could serve as an unnatural sugar containing a bioorthogonal Raman reporter for probing sialylated glycans. The close-to-perfect biocompatibility of isotopic substitution makes it attractive for studies where even small chemical modifications on glycans with azide or alkyne induce functional alterations.

In summary, we have developed a bioorthogonal Raman reporter strategy for probing cell surface glycans. This approach does not require the use of bioorthogonal reactions to chemically label the glycans with fluorescent probes. Rather, a Raman reporter is directly incorporated into cellular glycans for subsequent detection using SERS. This method possesses a high sensitivity, excellent signal specificity, and compatibility with a broad spectrum of Raman reporters. The alkyne and azide, two popular bioorthogonal reaction functional groups, can also be used as bioorthogonal Raman reporters. Novel Raman reporters such as nitrile and C–D have also be designed with improved properties. SERS imaging of glycans using this approach is currently under investigation. Notably, recent advances in developing high-speed slit-scanning confocal Raman microscopy should facilitate SERS imaging.[10,19] Furthermore, other Raman enhancing techniques such as coherent anti-Stokes Raman scattering (CARS)[20] and stimulated Raman scattering (SRS)[21] will further expand the utilities of Raman probes in studying glycosylation. These techniques do not require the enhancing substrate, and the SRS signal can be better quantified.[22] The sophisticated instrumentation, however, currently limits their accessibility.

Finally, this strategy may be further applied to detect proteins on cell surfaces. The alkyne and azide have been incorporated into proteins in living cells through metabolic or genetic approaches.[23] The SERS method with high detection sensitivity will be well-suited to study membrane proteins. Particularly, Raman vibration, similar to infrared vibration,[24] is sensitive to the local environment, which may be exploited to monitor the conformational changes of cell surface receptors.

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