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Senescence-associated sialidase revealed by an activatable fluorescence-on labeling probe†

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We report senescence imaging with a fluorescence-quenched self-immolative sialoside probe (Sia-RQ) which gives a reactive quinone methide to allow *in situ* fluorescence labeling of sialidases upon desialylation. Dramatic upregulation of lysosome-associated sialidase was uncovered in cell senescence with Sia-RQ, suggesting the use of sialidase as a new biomarker for senescence.

Cell senescence, a state of permanent cell cycle arrest, is crucial for tissue repair and antitumor defence.[1] Deregulated senescence has been linked to myriad pathological settings including ageing and diabetes,[1] promoting elimination of senescent cells to ameliorate ageing-related disorders.[2] As such approaches to image senescent cells would be of use to evaluate senescence-targeted biomedical interventions. Senescence-associated β-galactosidase has been used to detect cell senescence.[3] Although it is considered the current gold standard for senescence, β-galactosidase has also been detected in several cell types and selected cancers,[3e,4] necessitating additional biomarkers for senescence detection.

Sialic acids (Sia) are a family of anionic monosaccharides typically located at the termini of glycoconjugates on mammalian cell surfaces, whereby these terminal Sia residues are prone to sialidase-catalyzed hydrolysis.[5] Contrary to the roles of Sia-anchored on cell surfaces,[5] desialylation mediates a distinct set of biological processes ranging from pathogenicity to cell inflammation,[6] stimulating considerable efforts to image sialidases, mostly with Sia-masked fluorophores.[7] Although decreased levels of Sia have been observed on cell surfaces of aged erythrocytes relative to the young cells,[8] the correlation of desialylation with cell senescence has been largely unexplored. Inspired by these findings, we herein report the use of a fluorescence-quenched suicide substrate (Sia-RQ) of sialidases for senescence imaging. Sia-RQ features a self-immolative core of 4-hydroxymandelic acid integrated with a Sia entity for sialidase recognition, and a blackhole fluorescence quencher (BHQ) paired with rhodamine-X (ROX), a highly bright fluorophore suitable for cell imaging.[9] Upon desialylation, the fluorescence-quenched substrate self-immolates to yield a highly fluorescent and reactive quinone methide-ROX diad, which labels sialidases in live cells.

Activity-based chemical probes that could form covalent linkages with enzymes have been widely used in proteomics studies,[10] whereby hydrolases were often labeled with quinone methides generated upon enzymatic activation of rationally designed substrates.[11] To explore the correlation of sialidases with cell senescence, Sia-RQ, a quinone methide-releasing substrate, was synthesized for wash-free fluorescence-on sialidase imaging in live cells (Scheme 1).

Spectral analysis showed that the absorption band of the BHQ quencher substantially overlaps with the fluorescence emission of ROX (Fig. S1, ESI†), indicating fluorescence quenching of Sia-RQ *via* intramolecular fluorescence energy transfer. As expected, Sia-RQ exhibited strikingly lowered fluorescence emission relative to the ROX fluorophore and Sia-R, which is a structural analog of Sia-RQ lacking the BHQ entity (Fig. 1), with a quenching efficiency at 90%, indicating the potential of Sia-RQ for wash-free fluorescence-on imaging of sialidase activity.

We next evaluated the enzymatic processing of Sia-RQ *in vitro*. Incubation of recombinant α2-3,6,8-neuraminidase (referred to as sialidase) with 4-methylumbelliferyl-μ-D-*N*-acetylneuraminic acid (Sia-MU), a commercial substrate for sialidase, resulted in genesis and enhanced fluorescence of 4-methylumbelliferone (MU) over time (Fig. 2A), which is consistent with enzymatic hydrolysis triggered release of fluorescent 4-MU. In contrast, no enhancement

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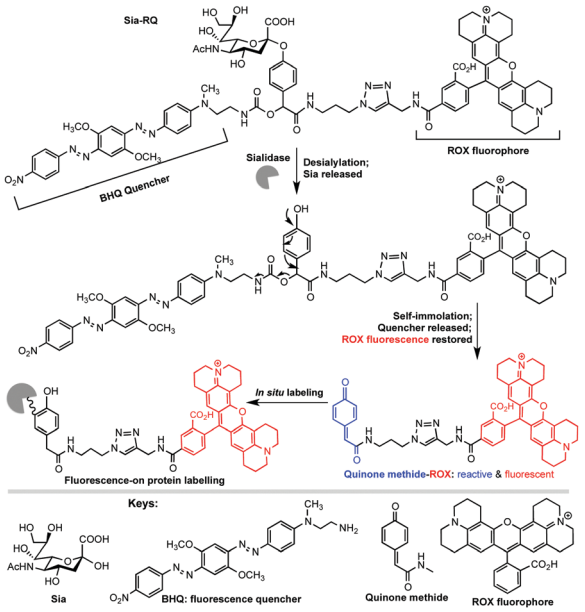
† Electronic supplementary information (ESI) available: Synthesis and analysis of Sia-RQ and Sia-R, imaging of intracellualr sialidases with Sia-MU, imaging of the specificity of Sia-RQ for lysosomes in diverse cell lines, time course analysis on sialidase imaging with Sia-RQ, and cytotoxicity of Sia-RQ. See DOI: 10.1039/c8cc07024e



Scheme 1 Fluorescence-on imaging of sialidase with Sia-RQ. Upon sialidase-mediated desialylation, fluorescence-quenched Sia-RQ undergoes self-immolation to give a highly fluorescent and reactive quinone methide-ROX diad, which *in situ* labels sialidase with a ROX fluorophore.

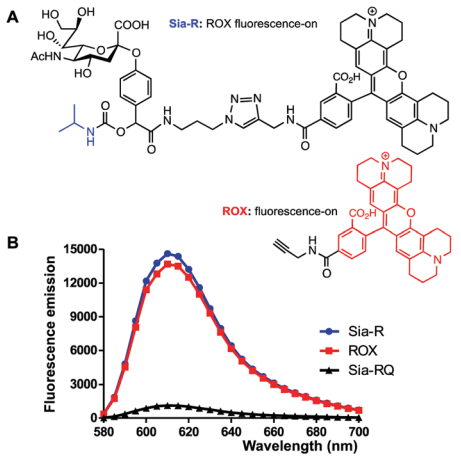


Fig. 1 Fluorescence quenching of Sia-RQ. (A) Fluorescence emission of sodium phosphate buffer (pH 7.4) containing 10 μM of Sia-RQ, Sia-R, or ROX. The fluorescence emission was measured using λ_{ex} = 580 nm. (B) Chemical structures of the ROX flluorophore and Sia-R free of the BHQ entity.

of ROX fluorescence was detected after incubating Sia-RQ with R-sialidase (Fig. 2B). This observation was initially surprising, as we expected ROX fluorescence to be restored upon desialylation of Sia-RQ owing to release of the BHQ quencher and abolished fluorescence-quenching (Scheme 1). The failed fluorescence activation of Sia-RQ by sialidase could originate from: (1) incapability of sialidase to hydrolyze Sia-RQ, (2) halted release of the BHQ quencher after desialylation, or (3) sialidase inactivation by *in situ* formed quinone methide. To clarify this discrepancy, sialidase precultured with Sia-RQ was further treated with Sia-MU. In sharp contrast to Sia-MU hydrolysis by sialidase exempt from Sia-RQ

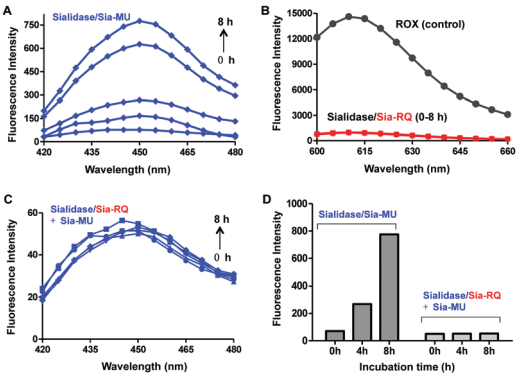


Fig. 2 Sialidase inactivation by Sia-RQ. Sia-MU (100 μM, (A)) or Sia-RQ (100 μM, (B)) was incubated with sialidase (300 units per ml) and the solutions were 10 fold diluted and then monitored for fluorescence emission over time using λ_{ex} = 365 nm for Sia-MU, and λ_{ex} = 580 nm for Sia-RQ. (C) Sialidase preincubated with Sia-RQ was further treated with Sia-MU. The solution was monitored for genesis of 4-MU. (D) Quantitative analysis on time course formation of 4-MU from Sia-MU (10 μM) in the presence of sialidase pretreated with or without Sia-RQ.

treatment (Fig. 2A), no catalytic formation of 4-MU was detected (Fig. 2C). In line with reported inactivation of glycosidases by quinone methides,[12] these results show that *in situ* generated quinone methide-ROX from Sia-RQ inactivates sialidase by covalent labeling of nucleophilic amino acid residues.

To confirm enzyme labelling, sialidase or bovine serum albumin (BSA) were incubated with Sia-RQ and then subjected to SDS-PAGE. Analysis of the gel revealed intense ROX fluorescence on dilute sialidase relative to low fluorescence in BSA which is likely contaminated with serum sialidase (Fig. 3). These results show covalent labeling of sialidase by Sia-RQ upon enzyme desialylation. In parallel, Sia-RQ was incubated with sialidase in the presence of BSA. Analysis revealed stronger ROX fluorescence on BSA, showing that *in situ* formed quinone methide-ROX could diffuse a distance and then be captured by surrounding proteins. Collectively, these results show that Sia-RQ is selectively activated by sialidase, which enables covalent sialidase labeling with the ROX fluorophore.

Activatable probes that release fluorophores in response to biological species are preferred for diverse applications such as low-background bioimaging and fluorescence activated cell sorting.[13] However, these studies are often limited by the tendency of the released fluorophores to diffuse or leak from the sites of bioactivation, stimulating extensive efforts to devise

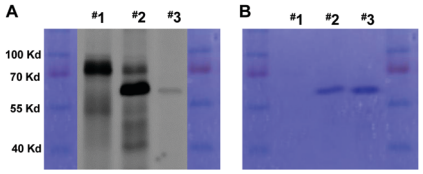


Fig. 3 Sialidase-triggered protein labeling with Sia-RQ. Sialidase (500 units, #1), sialidase (250 units)/BSA (0.5 μg) (#2), or BSA (1 μg, #3) was treated with Sia-RQ for 2 h and then subjected to SDS-PAGE. The gel was analyzed for fluorescence emission using λ_{ex} = 580 nm (A) or stained with Coomassie Brilliant Blue (B).

imaging agents stably trapped inside cells.[11a,14] Herein we show that Sia-RQ could be enzymatically activated to give a reactive dye-quinone methide intermediate that could be covalently trapped by sialidases or proteins in the vicinity. This would be beneficial to achieve long-term imaging or to pinpoint sialidases in varied biological settings.

With the demonstrated fluorecence-on protein labeling, Sia-RQ was applied to image endogenous sialidases in live cells. A panel of cell lines were cultivated with Sia-RQ and then visualized by confocal microscopy without cell washing. Intense ROX signals were observed inside A549, HeLa, HepG2, MCF-7 and U2OS cells whereas dim or no ROX fluorescence was identified in CHO, B16F10, and L929 cells. We observed punctate and intensive ROX fluorescence in HepG2 cells whereas ROX signals were rather plain in the cytosol of A549, HeLa, MCF-7 and U2OS cells. These imaging results suggest cell type-specific expression and biodistribution of sialidase. Flow cytometry analysis showed that sialidase-triggered ROX fluorescence becomes optimal in HepG2 cells at 2–10 h of incubation with Sia-RQ (Fig. S2, ESI†). In addition, the ROX fluorescence intensity in these cell lines largely correlates with that of 4-MU in cells stained with Sia-MU (Fig. S3, ESI†), confirming sialidase triggered ROX fluorescence inside cells.

To assess the influence of Sia-RQ on sialidase activity in live cells, HepG2 cells pretreated without or with Sia-RQ for 4 h were further cultivated with Sia-MU. Compared with the bright 4-MU fluorescence in HepG2 cells free of Sia-RQ, no fluorescence of 4-MU could be identified in HeLa cells pretreated with Sia-RQ (Fig. 4B). The incapability of Sia-RQ-treated cells to desialylate Sia-MU shows inactivation of intracellular sialidase by Sia-RQ, which is consistent with the aforementioned sialidase activation by Sia-RQ *in vitro* (Fig. 2D). Collectively these results prove the use of Sia-RQ as a fluorogenic suicide substrate to covalently label and inactivate sialidases in live cells.

Cell senescence is a stable state of growth arrest and has been implicated in myriad pathological settings. Although it is considered the current gold standard for senescence, β-galactosidase has also been detected in several cell types or cancers. Therefore it is of significant interest to develop complementary approaches for cell senescence imaging. Hence, we applied Sia-RQ to image cell senescence. Palbociclib has been reported to induce cell senescence in human liver cancer cell lines.[15] Huh7 cells, from human hepatocarcinoma, were thus treated with palbociclib for 0–14 days to induce senescence and then imaged by Sia-RQ. Confocal microscopy revealed that ROX fluorescence progressively increased at 3 d and leveled off at 7–14 d after palbociclib treatment (Fig. 5A and Fig. S4, ESI†). In control assays, Huh-7 cells imaged with Sia-MU displayed a similar tendency of MU fuorescence as Sia-RQ on progression of palbociclib-induced senescence (Fig. 5B). These results showed up-regulated sialidase activity during senescence.

We then performed dual staining of senescent cells with Sia-RQ and LysoTracker Blue, a dye specific for lysosomes. Confocal microscopy showed that the ROX signals were largely confined in the lysosomes (Fig. S5, ESI†). Of the four subtypes of sialidases in mammalian cells, neuraminidase-1 (Neu1) is specific for lysosomes. The imaging results suggest upregulation

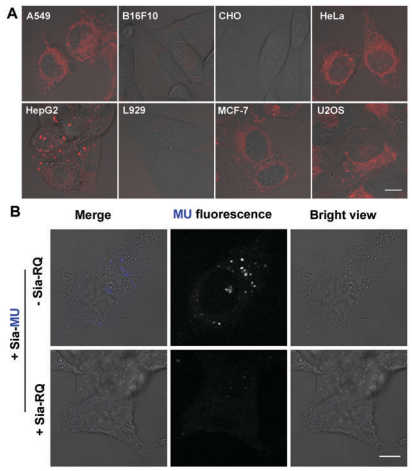


Fig. 4 Live cell imaging and inactivation of sialidases with Sia-RQ. (A) Imaging of sialidase by fluorescence-on ROX signals. The indicated cell lines were cultured with Sia-RQ and then visualized for intracellular ROX signals by confocal microscopy. Scale bar: 10 μm. (B) Inactivation of sialidase in cells with Sia-RQ. HepG2 cells precultured with or without Sia-RQ were further treated with Sia-MU and then imaged by confocal fluorescence microscopy for MU fluorescence. Scale bar: 10 μm.

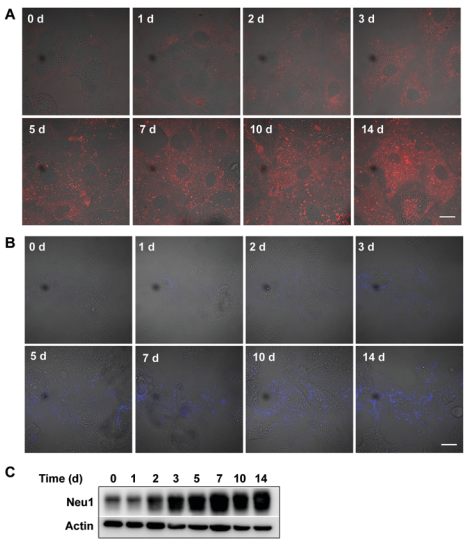


Fig. 5 Upregualtion of Neu1 in cell senescence revealed by Sia-RQ. Huh-7 cells were treated with palbociclib to trigger cell senescence. At the indicated time points after drug-treatment, the cells were cultivated with Sia-RQ (A) or Sia-MU (B), and then pinpointed for intracellular fluorescence by confocal microscopy. Scale bar: 20 μm. (C) Up-regulated expression of Neu1 confirmed by western blotting. Huh-7 cells were analyzed by western blotting for Neu1 expression at 0–14 days after treatment with palbociclib.

of Neu1 expression in senescence. To further confirm this finding, the senescent cells were analyzed by western blotting. Immunoblotting with Neu1 antibody revealed dramatically increased Neu1 expression in cells at 3–14 days after senescence induction (Fig. 5C), which is consistent with enhancement of sialidase activity observed in live cell imaging with Sia-RQ. Collectively, these results showed that lysosome-associated sialidase (Neu1) is strongly upregulated in the course of cell

senescence. Apart from desialylating cell surface glycoconjugates internalized into lysosomes, Neu-1 could relocate from the lysosomes into the plasma membrane to hydrolyze sialic acid residues of cell surface glycoconjugates.[6b,16] The lysosome-associated sialidase identified in senescent cells may decrease the sialylation status of the cells by these two distinct pathways.

Cell senescence is pivotal for a number of pathophysiological processes such as tissue repair, diabetes and age-related disorders, representing an interesting target for disease diagnosis and therapy. We herein devised a fluorescence-quenched sialoside (Sia-RQ) for fluorescence imaging of sialidase in live cells. Sia-RQ is prone to desilaylation triggered self-immolation, leading to release of the fluorescence quencher and concurrent formation of a highly reactive quinone methide–rhodamine diad to allow fluorescence labeling of sialidase in live cells. Dramatically enhanced expression of lysosome-associated sialidase (Neu1) is revealed in senescent cells by fluorescence imaging with Sia-RQ, and is further is confirmed by western blotting analysis. These findings validate the use of Sia-RQ for studying the roles of sialidases in diverse cellular events and also suggest the use of Neu1 as a new biomarker of cell senescence that could be targeted for imaging or therapeutic intervention.

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Conflicts of interest

There are no conflicts to declare.

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