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Intracellular click reaction with a fluorescent chemical Ca^{2+} indicator to prolong its cytosolic retention†

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The powerful strategy of "intracellular click reaction" was used to retain a chemical Ca^{2+} indicator in the cytosol. Specifically, a novel clickable Ca^{2+} indicator "N_3-fura-2 AM" was coupled with dibenzylcyclooctyl-modified biomacromolecules *via* copper-free click reaction in living cells and Ca^{2+} oscillation was observed for an extended period of time.

Changes in the intracellular concentration of calcium (Ca^{2+}) is an indispensable signal involved in the regulation of activities[1] such as neural transmission,[2,3] muscle contraction[4,5] and fertilization.[6] The recent development of Ca^{2+} indicators, such as small chemical fluorescent dyes or genetically encoded fluorescent proteins, has allowed various biological events related to Ca^{2+} signaling to be visualized.[7] Although genetic approaches based on Ca^{2+}-sensitive fluorescent proteins (*e.g.* cameleon,[8] G-CaMP[9]) have proved to be powerful tools for fluorescent microscopic observations in living animals, tissues or primary cultured cells, effective expression of these sensor proteins requires laborious steps such as production and maintenance of transgenic animals or viral vector preparation.[10] By contrast, small chemical Ca^{2+} indicators (*e.g.* fura-2,[11] fluo-3, rhod-2[12]) are easy to handle for staining tissue as well as cultured cells. However, these chemical Ca^{2+} indicators generally have too short cytosolic retention time for useful microscopic observations.[13] This problem arises because these indicators leak from the cell or accumulate in organelles due to the action of the anion transporters.[14,15] To date, two strategies to prolong the cytosolic retention of Ca^{2+} indicators have been reported. One strategy involves modification of the dye with cationic charges, which allow it to evade the activity of the anionic transporter. One such example is the commercially available cationic Ca^{2+} indicator fura-PE3,[16] although it still tends to accumulate in organelles during incubation. The second strategy involves conjugation of the dyes to macromolecules such as dextran.[17] However, due to the impermeability of the plasma membrane to dextran-conjugated dyes the troublesome microinjection technique is required for cell loading.

Here, we propose a convenient strategy to prolong the cytosolic retention by utilizing "intracellular click reaction" with cytosolic biomacromolecules such as proteins. We synthesized a fura-2 derivative "N_3-fura-2 AM" as a clickable Ca^{2+} indicator as shown in Fig. 1. Fura-2 AM is a ratiometric chemical Ca^{2+} indicator that allows the ratiometric measurement of Ca^{2+} concentration by dual excitation (λ_{Ex}, 340 nm/380 nm; λ_{Em}, 510 nm). N_3-fura-2 AM is capable of coupling with molecules containing an alkyne moiety *via* azide–alkyne 1,3-dipole cycloaddition (Huisgen reaction), which is an example of a "click reaction".[18] N_3-fura-2 AM also has an acetoxymethyl (AM) ester to improve its membrane permeability.[19] Compared with other intracellular labeling strategies, the click reaction has an advantage in terms of being rapid and bioorthogonal.[20] More recent advances in copper-free click chemistry using a cyclooctyne group allow us to perform click chemistry in the absence of copper catalysts, which are cytotoxic.[21] Thus we employed a copper-free click reaction to conjugate our Ca^{2+} indicator with biomacromolecules in the cytosol. This strategy circumvents the problem of leakage of the Ca^{2+}-indicator from the cell and allows the indicator to retain its function for an extended period of time.

We designed N_3-fura-2 AM bearing an azide moiety at the terminus of the alkyl chain so that the clickable site is maintained at a distance from the chelating ligand that binds Ca^{2+}. N_3-fura-2 and N_3-fura-2 AM were synthesized from *p*-hydroquinone through 14 and 15 steps (see ESI†), respectively.

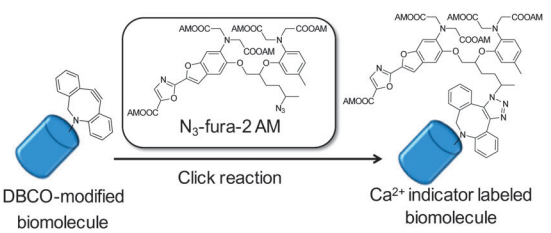

Fig. 1 Chemical structure of N_3-fura-2 AM and schematic illustration of the copper-free click reaction with dibenzylcyclooctyl (DBCO)-modified biomolecules.

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The critical step in the synthetic procedure was deprotection of the benzyl group of the precursor. Deprotection by catalytic reduction was deemed unsuitable because the azide group was likely to be reduced. Thus, we chose a deprotection method using $MgBr_2$, which is based on the selective cleavage of an aromatic benzyl group at the *ortho* position to a carbonyl group.[22]

The Ca^{2+} sensitive excitation spectrum of N_3-fura-2 (Fig. 2b) was similar to that of fura-2 (Fig. S2, ESI†). The affinity of N_3-fura-2 for Ca^{2+} (K_d = 304 nM at pH 7.2, 28 °C) was relatively low by comparison to that of conventional fura-2 (K_d = 125 nM under the same conditions). We assume that the alkyl chain attachment might affect the flexible region of the Ca^{2+} chelating ligand.[23]

In order to evaluate the clickability of N_3-fura-2 (AM), we prepared bovine serum albumin (BSA) modified with dibenzylcyclooctyl (DBCO) as a copper-free click reagent.[24] DBCO-modified BSA was prepared using DBCO succinimidyl ester, which was non-selectively conjugated to amine groups of BSA. DBCO-modified BSA and N_3-fura-2 AM (10 μM) were mixed in an aqueous buffer solution. We checked how the click reaction proceeded in the sample tube by SDS-PAGE analysis (Fig. 2a). After mixing DBCO-modified BSA and fura-2 AM under the same conditions, fluorescence was barely observable at the band of BSA stained with Coomassie Brilliant Blue. This result suggests that the fluorescence labeling of BSA was caused by coupling of N_3-fura-2 AM and DBCO-modified BSA *via* the copper-free click reaction. There was no non-specific binding of fura-2 to BSA.

We further investigated the influence of the BSA conjugation on the Ca^{2+}-indicating function of the fura-2 moiety by comparison to that of free N_3-fura-2. As shown in Fig. 2c, both the excitation spectrum and the affinity after click reaction with DBCO-modified BSA (K_d = 298 nM) were almost identical to those before the reaction (K_d = 304 nM). This finding suggests that macromolecules such as BSA conjugated by click reaction do not affect the properties of the fluorophore and the environment surrounding the chelating structure of fura-2.

Next, we performed the intracellular click reaction in HeLa cells by using N_3-fura-2 AM. Firstly, the intracellular environment inside HeLa cells was non-specifically modified with DBCO succinimidyl ester. N_3-fura-2 AM or fura-2 AM was then applied to DBCO modified HeLa cells using the same procedure (1 μM loading solution was incubated with cells for 30 min at 37 °C). The fluorescence microscopic images of N_3-fura-2 stained cells at 5 min after washing (Fig. 3a) showed the fluorescence intensity of the nucleus was

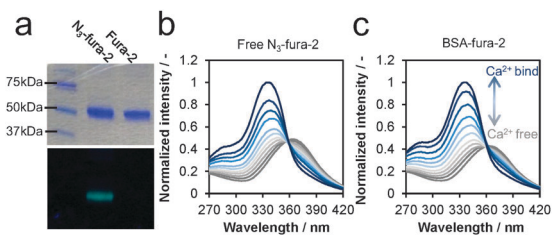

Fig. 2 Identification of N_3-fura-2 coupled DBCO-modified BSA. (a) SDS-PAGE analysis of DBCO-modified BSA coupled with N_3-fura-2 and the mixture of DBCO-modified BSA with fura-2. The gel was imaged whilst being (top) stained with Coomassie Brilliant Blue (CBB) and (bottom) irradiated with UV at 365 nm. Excitation spectra of N_3-fura-2 at different Ca^{2+} concentrations (b) before and (c) after BSA conjugation.

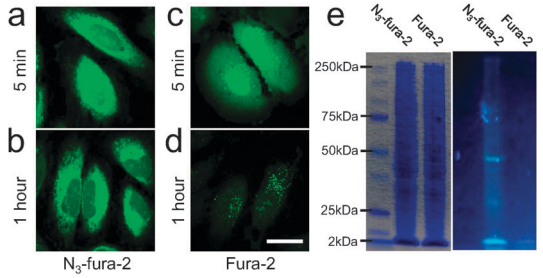

Fig. 3 Confirmation of intracellular click reaction of N_3-fura-2 with DBCO-modified biomacromolecules. Fluorescence images of HeLa cells ((a and b) N_3-fura-2 and (c and d) fura-2 at 5 min and 1 hour after washing, respectively). The scale bar is 20 μm. (e) SDS-PAGE analysis of the lysate after intracellular click reaction with N_3-fura-2. The fura-2 dye was added to the cells after DBCO modification treatment. The gel was (left) stained with CBB and (right) irradiated with UV at 365 nm.

very weak compared with that of the cytosol. In contrast, for fura-2 the difference in the intensities between the cytosol and the nucleus was very small (the detailed description of the intracellular localization of N_3-fura-2 is provided in ESI†). These observations imply that N_3-fura-2 was conjugated with cytosolic or membrane proteins, thereby preventing the fluorescent dye from penetrating through the nuclear pores. The SDS-PAGE analysis of lysates from N_3-fura-2 stained cells showed many fluorescent bands in the entire lane and indicated the conjugation between N_3-fura-2 and various intracellular proteins (Fig. 3e). Therefore, the fluorescence intensities in the case of N_3-fura-2 should have originated from the intracellular click reaction of N_3-fura-2 with intracellular DBCO-modified proteins.

Finally, we evaluated the cytosolic retention time of N_3-fura-2 by comparison to those of fura-2 and a cationic long-term Ca^{2+} indicator (fura-PE3). Fig. 4a and b shows a series of fluorescence images of Ca^{2+} indicator (N_3-fura-2, fura-2) loaded HeLa cells (images of fura-PE3 are shown in Fig. S6, ESI†). As expected, N_3-fura-2 after intracellular click reaction remained in the cytosol for a longer period of time than fura-2 and fura-PE3. Quantitative analyses of the cytosolic retention of the fluorescence signal indicated a significant difference between conventional Ca^{2+} indicators and N_3-fura-2 (p value = 0.003 between N_3-fura-2 and fura-PE3 at 180 min after loading) (Fig. 4c). The time taken for the fluorescence to decrease to half the initial level for fura-2, fura-PE3 and N_3-fura-2 was 35 min, 63 min and 185 min, respectively. For short term measurements, leakage of dye into the medium might cause baseline drift, regardless of whether or not ratiometric methods are employed. Indeed, during microscopic observation without stimulation of cells, the apparent Ca^{2+} dependent fluorescence ratio of fura-2 did drift (Fig. 4d). By contrast, N_3-fura-2 after conjugation with intracellular macromolecules did not show such a drift, thereby enhancing the accuracy of the measurements. In addition, to determine the duration of effective dye retention, we checked whether indicators can work with ligand (histamine) mediated Ca^{2+} oscillation at 5 min, 3 hours and 6 hours after dye loading. At 5 min after loading, the fluorescence ratio of both fura-2 and N_3-fura-2 indicated similar transient Ca^{2+} elevation, showing little influence of DBCO and N_3-fura-2 conjugation on the Ca^{2+} oscillation and its detection. However, in the case

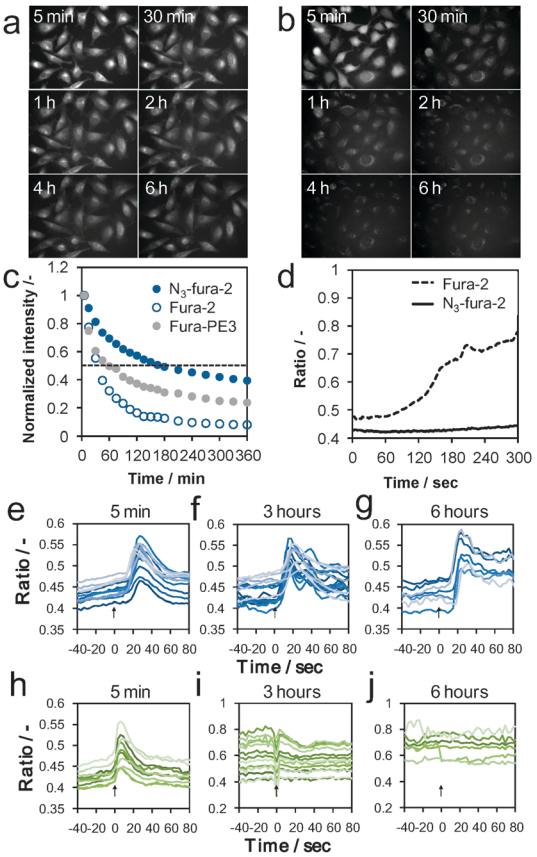

Fig. 4 Evaluation of intracellular retention time of the Ca^{2+} indicators. The fluorescence images of N_3-fura-2 (a) and fura-2 (b) were taken after 6 hours. (c) Time course of the changes of fluorescence intensity emitted from fura-2, fura-PE3, and N_3-fura-2 loaded HeLa cells was monitored. (d) Baseline drift of the fluorescence ratio (F_{340}/F_{380}) of fura-2 and N_3-fura-2 during a period of 5 min. (e–j) Long-term changes in Ca^{2+} transients by histamine detected as the fluorescence ratio recorded at 5 min, 3 hours and 6 hours after loading of N_3-fura-2 (e–g) and fura-2 (h–j), respectively. The addition of histamine is marked by an arrow at $t = 0$.

of fura-2 this phenomenon could not be observed 3 hours and 6 hours after loading because the signal was lost in the background noise (Fig. 4h–j). By contrast, fluorescence ratio changes of N_3-fura-2 including cytosolic Ca^{2+} oscillation could even be observed 6 hours after loading with a high signal-to-noise ratio (Fig. 4e–g). These results clearly indicated that N_3-fura-2 was retained within the cytosol and acted as an effective indicator for at least 6 hours.

In conclusion, we have synthesized a novel clickable Ca^{2+} indicator and achieved "intracellular copper-free click reaction" with DBCO-modified intracellular proteins. Our Ca^{2+} indicator displays an extended cytosolic retention by comparison to fura-2 and commercially available long-term Ca^{2+} tracer fura-PE3. Although the retention time is extended to 6 hours, it is shorter than the successive expression of fluorescent proteins of several days.[25] The advantage of our method is unnecessity of any troublesome gene transfection steps. Just the addition of the DBCO reagent and the indicator to the cells is enough to measure cytosolic Ca^{2+} oscillation. Development of this method for long-term Ca^{2+} tracing will be invaluable in studying transient changes in the intracellular concentrations of Ca^{2+} during various biological processes, including the circadian rhythm[25] and embryonic development,[26] as well as *in vivo* Ca^{2+} imaging. Even during short-term measurements of intracellular Ca^{2+} levels, leakage and compartmentalization of the indicator can preclude accurate Ca^{2+} measurements. More recently, click labeling methods for sugars,[27] lipids,[28] glycans,[29] nucleic acids[30] and other metabolites bearing an alkyne moiety have been reported. Thus, our strategy can be applied to various cellular components bearing alkyne or cyclooctyne moieties. In such cases, the chemical Ca^{2+} indicator will be located at the position of interest.

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