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Synthesis of an azido-tagged low affinity ratiometric calcium sensor

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ABSTRACT

Changes in high localised concentrations of Ca^{2+} ions are fundamental to cell signalling. The synthesis of a dual excitation, ratiometric calcium ion sensor with a K_d of 90 μM, is described. It is tagged with an azido group for bioconjugation, and absorbs in the blue/green and emits in the red region of the visible spectrum with a large Stokes shift. The binding modulating nitro group is introduced to the BAPTA core prior to construction of a benzofuran-2-yl carboxaldehyde by an allylation–oxidation–cyclisation sequence, which is followed by condensation with an azido-tagged thiohydantoin. The thiohydantoin unit has to be protected with an acetoxymethyl (AM) caging group to allow CuAAC click reaction and incorporation of the KDEL peptide endoplasmic reticulum (ER) retention sequence.

1. Introduction

Free Ca^{2+} is an important cell messenger involved in numerous processes such as muscle contraction, cell division and cell death.[1] The effects of changing the Ca^{2+} concentration, [Ca^{2+}], can have a wide reach, extending both within and among cells.[1] [Ca^{2+}] can also affect a range of different activities by acting selectively as a highly localised signal operating in subcellular regions. The free cytosolic [Ca^{2+}] is typically in the region of 100 nM at rest and increases to an averaged peak cellular value of <1 μM when the cell is activated.[2] However, to selectively perform multiple functions, cells localise signals to certain regions by creating high local [Ca^{2+}] in the range of tens to several hundred micromolar.[3] These localised concentrations of Ca^{2+} enable the control of specific cellular activities, such as ion channel and transcription factor activation. In addition to high local concentrations in the cytoplasm, organelles such as the mitochondria, sarcoplasmic reticulum, endoplasmic reticulum and Golgi apparatus each store Ca^{2+} at concentrations (hundreds of micromolar) far above the cytoplasmic average.[4] Understanding of how [Ca^{2+}] selectively controls cell function remains preliminary because of the difficulties in studying Ca^{2+} signals in specific cell regions. Therefore, there have been efforts to develop sensors to study localised and dynamic calcium concentrations.[5–7]

As part of these efforts, we set out to develop a fluorescent sensor that would detect changes in the high [Ca^{2+}] found in subcellular stores. We wanted a sensor that absorbed and emitted in the visible rather than UV region of the spectrum so that its use would disturb the system minimally, and that was ratiometric so that quantification was straightforward. Above all, we wanted to incorporate a tag so that bioorthogonal chemistry could be used to attach a targeting group or to attach the sensor to biomolecules.

We chose to develop a sensor using the highly selective octadentate binding of the Ca^{2+} ion by a 1s,2-bis(*ortho*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) ligand,[8,9] which has become a powerful tool for studying changes in [Ca^{2+}].[10–18] Recent work has focused on developing sensors that absorb and emit in the low-energy red and near infrared regions of the spectrum,[14–17] both to limit damage and to allow the use of several different fluorophores in the same sample (multiplexing).[18] In line with this, we decided that our BAPTA-based sensor should absorb and emit in the red region of the spectrum.

We considered an ideal Ca^{2+} sensor would be fluorescent both with Ca^{2+} bound and in its unbound state, but with different absorption or emission wavelengths. This would allow the [Ca^{2+}] to be determined directly using the ratio of the two forms using the binding dissociation constant of the sensor (K_d). The use of such so-called ratiometric probes offers many advantages over traditional

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off/on probes such as correction for artefacts e.g., photobleaching and variation in probe loading.[19] However, the development of ratiometric Ca^{2+} sensors has lagged behind the development of the traditional off/on probes. Indeed, most biological studies using this technique[20,21] rely on the original two sensors, Fura-2 and Indo-1,[8,9] which both absorb in the UV region of the spectrum. There are recent promising ratiometric probes developed by Liu et al.,[13] but the area is underdeveloped. On the other hand, we and a few others have used Fura-Red[22,23] as a ratiometric Ca^{2+} sensor that is excited by visible light. It is a dual excitation ratiometric probe so that $[Ca^{2+}]$ can easily be determined from the ratio of the emissions at 640 nm when excited at 436 nm (Ca^{2+}-bound sensor) and when excited at 472 nm (free sensor).[23] Fura-Red's very large Stokes shift of ~200 nm also allows the simultaneous use of other dyes such as Fluo-4.[24]

Given these excellent properties of Fura-Red, we decided to adjust its binding affinity so that it could detect changes when $[Ca^{2+}]$ is high. The binding dissociation constant (K_d) should be midway between the starting and final $[Ca^{2+}]$ in the process under study to maximise the observable response of the sensor. Most of the commonly-used Ca^{2+} sensors have K_d values within the nanomolar range (e.g., Fura-Red has a K_d=380 nM[22,25]), which makes them suitable for measuring global cytosolic $[Ca^{2+}]$ fluxes.[10,11] We reasoned that a low affinity Fura-Red derivative, NitroAzidoFuraRed (Fig. 1), could be prepared that would have an azido tag for bioorthogonal chemistry.[26]

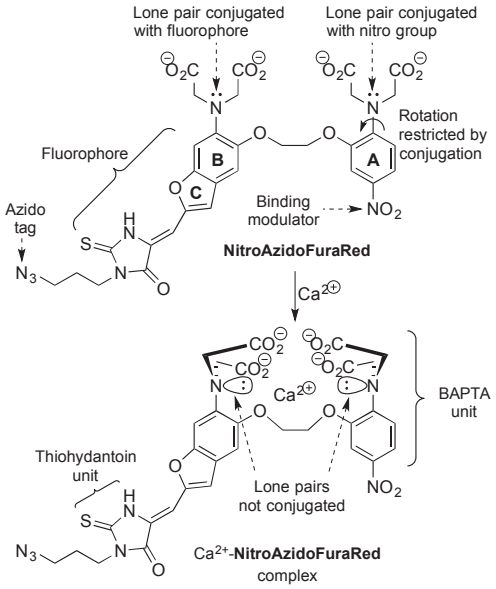


Fig. 1. The design of NitroAzidoFuraRed.

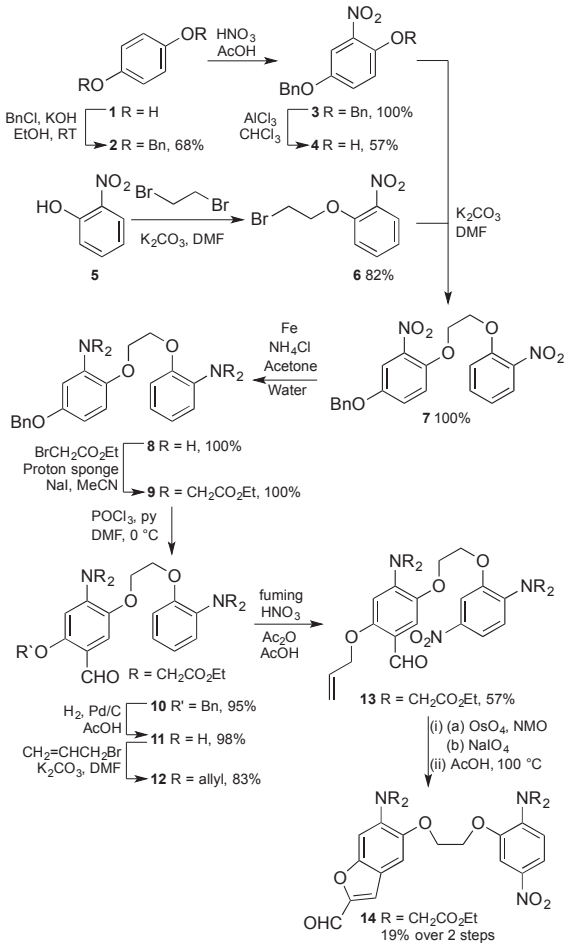
Fura-Red's fluorescent properties arise from photoinduced charge transfer because the fluorophore is in direct conjugation with the BAPTA unit and that conjugation changes when the nitrogen lone pair turns out of plane during binding.[18] We reasoned that the binding affinity would be decreased by incorporating an electron-withdrawing group on the A ring, which would increase the conjugation with the amino group.[18] This in turn would disfavour the rotation out of plane that is required for binding to Ca^{2+} and so lower the binding affinity, raising K_d.[11] We wished to generate a ratiometric probe with a K_d in the 50–100 μM range that could be used to investigate intra-organellar $[Ca^{2+}]$. Having considered the binding affinities of known Ca^{2+} sensors[10,11,27] and correlations with Hammett σ constants,[28] we decided to incorporate a nitro group in the BAPTA core.[29]

We also decided to incorporate an azido tag in NitroAzidoFuraRed because it would offer a universal site of attachment by bioorthogonal chemistry[26] that could be used to incorporate a targeting group or biomolecule using copper-catalysed or strain-promoted azide–alkyne cycloaddition (CuAAC[16,27,30] or SPAAC[31]). The azido group would be attached through the thiohydantoin unit so that it would be distal from the BAPTA binding site to minimise potential interference with Ca^{2+} binding.[16,27,30,31]

In summary, we had designed NitroAzidoFuraRed to be a low-affinity, ratiometric, Ca^{2+} sensor excitable with visible light and displaying a large Stokes shift, which would have the potential for attachment to targeting groups and biomolecules. Herein, we show how it was synthesised and present its calcium ion binding properties. We also provide the first examples of CuAAC on thiohydantoin derivatives.

2. Results and discussion

There have been no published syntheses of low affinity Fura-Red indicators. Our approach was to construct the BAPTA core **8** by adapting the route of Grynkiewicz et al.[9] Starting from hydroquinone **1**, benzyl protection gave bisether **2**, which was then converted into the nitro derivative **3**. Selective deprotection of the *ortho*-benzyl group was achieved using aluminium trichloride instead of TFA to give phenol **4** in good yield.[32] Coupling phenol **4** with known bromide **6**, prepared from phenol **5**,[33] gave bis-nitro compound **7** in quantitative yield. Reduction of the nitro groups in the presence of the benzyl ether was achieved using iron rather



Scheme 1.