Imaging Agents

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Ubiquinone-Rhodol (UQ-Rh) for Fluorescence Imaging of NAD(P)H through Intracellular Activation**

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Abstract: *The nicotinamide adenine dinucleotide (NAD) derivatives NADH and NADPH are critical components of cellular energy metabolism and operate as electron carriers. A novel fluorescent ubiquinone-rhodol derivative (UQ-Rh) was developed as a probe for NAD(P)H. By using the artificial promoter $[(\eta^5\text{-}C_5Me_5)Ir(phen)(H_2O)]^{2+}$, intracellular activation and imaging of NAD(P)H were successfully demonstrated. In contrast to bioorthogonal chemistry, this "bioparallel chemistry" approach involves interactions with native biological processes and could potentially be used to control or investigate cellular systems.*

The nicotinamide adenine dinucleotide (NAD) derivatives NADH and NADPH (general term: NAD(P)H) play an important role in cellular metabolism and energy systems as electron carriers.[1] NADH is generated by the tricarboxylic acid (TCA) cycle, with three NADH molecules produced per cycle. The phosphorylated form of NADH, NADPH, is involved in photosynthesis and in the Entner–Doudoroff pathway[2] of glycolysis.[3] NADH has also been investigated as a therapeutic agent for the treatment of Alzheimer's[4] and Parkinson's diseases.[5]

Several imaging methods for intracellular NAD(P)H, including an autofluorescence-based method,[6] have been investigated. However, those methods are implemented using ultraviolet (UV) light, thus resulting in low sensitivity and interference by intracellular materials. A green fluorescent protein (GFP)-based technique for imaging the NADH/NAD^+ ratio has also been developed.[7] In that case, the mechanism of sensing depends on conformational variation of the protein and requires intracellular gene expression. Although several other methods for NAD(P)H determination exist, including enzymatic reaction kits,[8] electrochemical methods, and chemosensors, these have been developed for use in vitro and are not suitable for cellular use.[9] To date, molecular-probe-based imaging of intracellular NAD(P)H has not been reported.

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The activation of biological molecules (e.g., acetyl CoA) in cells by using an artificial promoter and promoter-assisted molecular imaging has been proposed.[10] This approach has been adopted here as an imaging strategy that can be applied to NAD(P)H.

It has been reported that NAD(P)H can be activated in vitro by using an Ir complex,[11] although this method has not been applied in an intracellular context. On the other hand, a quinone-based molecular probe (indolequinone derivative) has been reported.[12] We hypothesized that a ubiquinone derivative might react with NAD(P)H based on its biological role and reduction potential. Furthermore, enhanced sensitivity can be obtained by using an Ir-complex-based artificial promoter, which can activate NAD(P)H. Therefore, we designed the ubiquinone-rhodol conjugate UQ-Rh, which contains ubiquinone as an NAD(P)H-reactive site and the rhodol fluorophore as a novel biocompatible fluorescent probe, the fluorescence emission of which occurs in the visible region (Figure 1).[13]

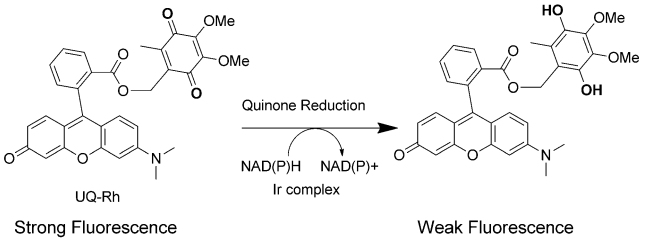

Figure 1. The reduction of UQ-Rh by NAD(P)H.

UQ-Rh was synthesized from a ubiquinone derivative and rhodol (see the Supporting Information). It possesses an absorbance maximum at 492 nm ($\varepsilon = 1.66 \times 10^4\ cm^{-1}M^{-1}$) and a fluorescence maximum at 518 nm with a quantum yield of 0.733 in PBS buffer at pH 7.4 (Figure 2, blue lines).

To observe the fluorescence response as a result of quinone reduction, UQ-Rh was dissolved in PBS buffer and the quinone substituents were reduced by using $Na_2S_2O_4$.[14] Following reduction, the fluorescence dropped to 1/30 of the starting value (Figure 2, red lines). The attenuation of the fluorescence is considered to be due to intramolecular photoinduced electron transfer (PET)[15] from hydroquinone to rhodol.

The NADPH fluorescence response of UQ-Rh was measured with no promoter, in the presence of a promoter (1 mM $[(\eta^5\text{-}C_5Me_5)Ir(phen)(H_2O)]^{2+}$), or in the presence of 2 $\mu g\,mL^{-1}$ quinone reductase in PBS.

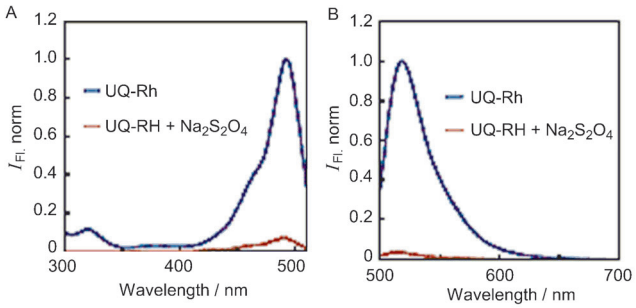

Figure 2. A) Fluorescence excitation spectra (detected at 520 nm), and B) fluorescence emission spectra (excited at 488 nm) of UQ-Rh in the presence and absence of $Na_2S_2O_4$ in PBS (pH 7.4). The normalized fluorescence intensity was plotted.

The fluorescence emission intensity of UQ-Rh decreased in 1 mM NADPH. Moreover, in the presence of the promoter $[(\eta^5\text{-}C_5Me_5)Ir(phen)(H_2O)]^{2+}$, a 4-fold enhancement of the reaction rate was observed and UQ-Rh was considered to be effectively reduced. However, quinone reductase was not so effective (1.45-fold enhancement), probably because of the poor fit of UQ-Rh to the pocket of this enzyme (Figure S1 in the Supporting Information).

The dependence of the UQ-Rh response on NADPH concentration was determined in the presence of 0.5 mM $[(\eta^5\text{-}C_5Me_5)Ir(phen)(H_2O)]^{2+}$ (Figure 3). A weak response was

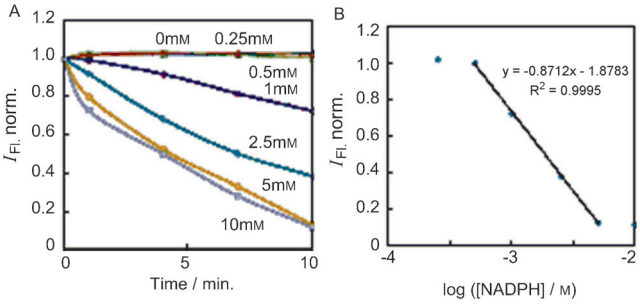

Figure 3. A) The time-dependence and dose-dependence (in terms of NADPH) of the fluorescence intensity (normalized) of UQ-Rh in the presence of 0, 0.25, 0.5, 1.0, 2.5, 5, and 10 mM of NADPH (10 μM UQ-Rh, 0.5 mM $[(\eta^5\text{-}C_5Me_5)Ir(phen)(H_2O)]^{2+}$, PBS pH 7.4).

first observed after the addition of 0.5 mM NADPH. At 10 mM NADPH, an 8.6-fold decrease in fluorescence intensity was observed after 10 min. By preparing a calibration curve for fluorescence intensity after the addition of NADPH in the presence of 0.5 mM $[(\eta^5\text{-}C_5Me_5)Ir(phen)(H_2O)]^{2+}$, a linear correlation was found between 0.5 mM and 5 mM NADPH. NADPH can thus be quantitatively determined in this concentration range. Because of its response to NADPH, UQ-Rh can be used to quantify NADPH with a sensitivity that can be enhanced by adding the Ir complex as a promoter.

The NADH response was also measured and compared with the response to NADPH. NADH produced a decrease in fluorescence intensity similar to that observed for NADPH. In the presence of 0.5 mM $[(\eta^5\text{-}C_5Me_5)Ir(phen)(H_2O)]^{2+}$, the reaction of NADPH was 1.7-fold faster than of NADH (Figure S2). This difference may be due to the presence of the negatively-charged phosphate group in NADPH, which likely strengthens the interaction of NADPH with the positively-charged Ir complex. Overall, the response difference between NADPH and NADH is not so large.

The selectivity of UQ-Rh for NAD(P)H over potentially interfering intracellular apecies was also investigated. Intracelluar cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) and glutathione (at intracellular concentrations) gave no fluorescence response with UQ-Rh (Figure S2). Furthermore, we applied the fluorescent probe UQ-Rh and the promoter $[(\eta^5\text{-}C_5Me_5)Ir(phen)(H_2O)]^{2+}$ to the intracellular activation and imaging of NAD(P)H in HeLa cells. UQ-Rh was introduced by incubating UQ-Rh with the HeLa cells. Fluorescence-microscopy imaging revealed that although some UQ-Rh seemed to be localized in organelles, the majority of the UQ-Rh appeared to stain the cytosol.

The addition of pyruvate (5 mM), which activates the TCA cycle in cells and increases the intracellular NADH concentration, resulted in a decrease in cellular fluorescence, thus indicating that UQ-Rh can indeed be used to measure NADH increases in cells simply by monitoring fluorescence (Figure S3).

The promoter $[(\eta^5\text{-}C_5Me_5)Ir(phen)(H_2O)]^{2+}$ was added to the UQ-Rh stained HeLa cells and the time dependence of the fluorescence intensity was imaged. Prior to the addition of $[(\eta^5\text{-}C_5Me_5)Ir(phen)(H_2O)]^{2+}$, a slight decrease in fluorescence was observed. This result is considered to be due to the reaction of NAD(P)H occurring at resting levels in the cells. Upon the addition of 0.5 mM $[(\eta^5\text{-}C_5Me_5)Ir(phen)(H_2O)]^{2+}$, an increase in fluorescence was observed (probably owing to injection shock) with a subsequent rapid decrease in fluorescence intensity (Figure 4 A). Hydride was thus transferred

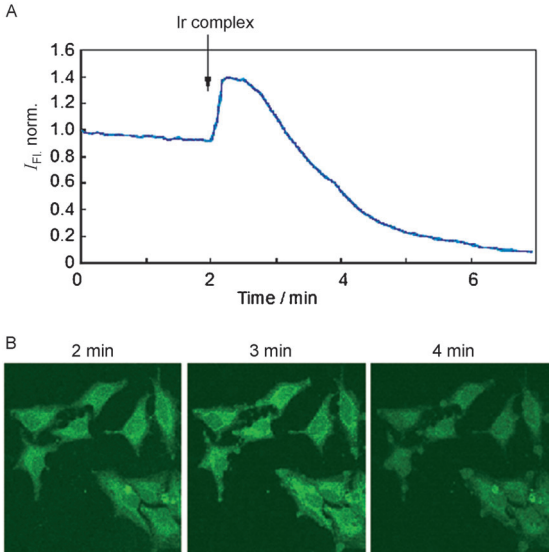

Figure 4. Fluorescence imaging of HeLa cells. A) Normalized fluorescence intensity before and after addition of the Ir complex $[(\eta^5\text{-}C_5Me_5)Ir(phen)(H_2O)]^{2+}$ (0.5 mM) at 2 min. ([UQ-Rh] = 10 μM, excitation at 488 nm; average of 9 cells). B) Fluorescence images (excitation at 488 nm).

from NAD(P)H to the Ir complex by the artificial promoter and could be imaged by observing the fluorescent probe.

Further observation of the cells following NAD(P)H activation revealed that 4 min after the addition of $[(\eta^5\text{-}C_5Me_5)Ir(phen)(H_2O)]^{2+}$, leaching of the cell contents occurred, which was followed after 7 min by cell death. NAD(P)H activation induced ubiquinone reduction or an NAD(P)H shortage in the cells might have affected cell homeostasis, thus resulting in cell necrosis (Figure 4B).

The ubiquinone-rhodol-derived fluorescent probe UQ-Rh responds to NADPH with an 8.6-fold decrease in fluorescence intensity over 10 min in the presence of $[(\eta^5\text{-}C_5Me_5)Ir(phen)(H_2O)]^{2+}$. UQ-Rh was introduced into HeLa cells and, following the addition of $[(\eta^5\text{-}C_5Me_5)Ir(phen)(H_2O)]^{2+}$, the activation of NAD(P)H could be imaged by observing the decrease in fluorescence. Although the use of this probe for the quantification of intracellular NAD(P)H is not easy, we have demonstrated the first use of an artificial fluorescent probe for the activation and imaging of NAD(P)H in cells. The activation of NAD(P)H induces necrosis by inducing errors in cell homeostasis.

The activation of molecules within cells can induce novel chemical reactions, an approach that contrasts with the conventional concept of bioorthogonal chemistry[16] in the field of chemical biology. Bioorthogonal chemistry denotes chemical reactions that occur without interfering with native biological process. By contrast, in our system, interactions do occur (i.e. activation of NAD(P)H). Considering these interactions, we hope to investigate novel aspects of living systems by applying activation and functionalization within cells to influence their intracellular metabolic processes. To distinguish this from the widely known bioorthogonal reactions, we have applied the label "bioparallel chemistry" and believe that it may be possible to use this strategy to control cellular systems or living systems in general. This system could form the basis of a new approach in medicine, with the development of novel artificial promoters and reagents.

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