

Short Communications

## Electrochemical Evaluation of Nitroxyl Radical Catalysts and Electrochemical Detection of Cyclosporin A

Sachiko Komatsu<sup>1</sup>, Yusuke Sasano<sup>2</sup>, Kyoko Sugiyama<sup>1</sup>, Kazuhiro Watanabe<sup>1</sup>, Masayuki Kumano<sup>2</sup>, Kentaro Yoshida<sup>3</sup>, Tetsuya Ono<sup>3</sup>, Yoshiharu Iwabuchi<sup>2</sup>, Tsutomu Fujimura<sup>1</sup>, Katsuhiko Sato<sup>1,4,\*</sup> and Yoshitomo Kashiwagi<sup>3,\*</sup>

<sup>1</sup> Faculty of Pharmaceutical Science, Tohoku Medical and Pharmaceutical University, 4-4-1 Komatsushima, Aoba, Sendai, Miyagi 981-8558, Japan;

<sup>2</sup> Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan;

<sup>3</sup> School of Pharmaceutical Sciences, Ohu University, 31-1 Misumido, Tomita-machi, Koriyama, Fukushima 963-8611, Japan;

<sup>4</sup> Department of Creative Engineering, National Institute of Technology, Tsuruoka College, 104 Sawada, Inooka, Tsuruoka, Yamagata 997-8511, Japan;

\*E-mail: [y-kashiwagi@pha.ohu-u.ac.jp](mailto:y-kashiwagi@pha.ohu-u.ac.jp)

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Nitroxyl radical catalysts oxidize alcohols under an applied electric potential. It is possible to quantify the alcohol concentration from the resulting oxidation current. In this work, we evaluated the catalytic activity of nitroxyl radicals (or their corresponding hydroxylamines), including 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) as representative nitroxyl radicals, acetoamido-TEMPO, which shows higher oxidation potential than TEMPO owing to the acetoamido group, AZADO, Nor-AZADO, and NNO as less-hindered bicyclic nitroxyl radicals, and NHPI as an *N,N*-diacyl-type hydroxylamine, in acetonitrile solution. TEMPO, AZADO and NNO were also evaluated for their ability to oxidize alcohols in organic solvents, and their reactivity was compared with the electrochemical response. The most active NNO was used for electrochemical detection of cyclosporin A, a drug with a hydroxyl group. An abstract stating the features and drawing attention to the novel aspects.

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**Keywords:** nitroxyl radical; TEMPO; cyclosporine A; sensor

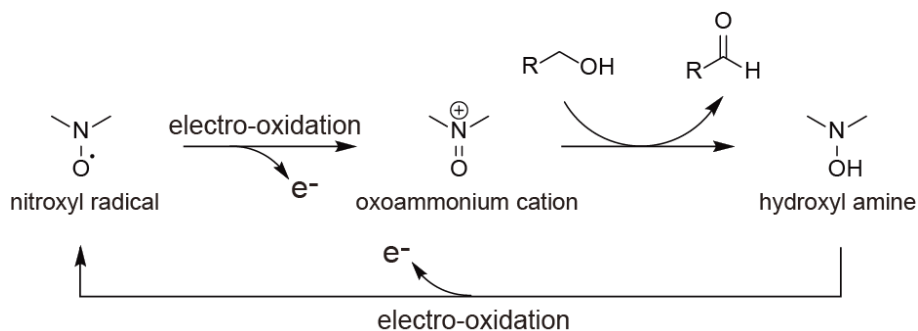
### 1. INTRODUCTION

Cyclosporin A (CSA), a lipophilic, cyclic peptide containing 11 amino acids [1], has been widely used to suppress rejection in organ transplantation. CSA binds to the T-cell receptor protein cyclophilin,

and this complex acts as a calcineurin inhibitor that suppresses cytokine production. CSA is also applied therapeutically to treat autoimmune diseases such as Behcet's disease, aplastic anemia, and nephrotic syndrome. Since CSA has a narrow effective treatment range, determination of the blood CSA levels is required in order to avoid the risk of rejection or organ toxicity. Therefore, therapeutic drug monitoring (TDM) is typically performed during immunosuppressive therapy with CSA.

Many TDM methods for CSA are carried out by immunoassay or liquid chromatography-tandem mass spectrometry (LC/MS/MS), each of which has advantages and disadvantages. Major commercially available kits for determination of CSA levels in whole blood have been developed based on immunoassays, such as the enzyme multiplied immunoassay technique (EMIT), affinity column mediated immunoassay (ACMIA) [2], chemiluminescence immunoassay (CLIA) [3], and electrochemiluminescence immunoassay (ECLIA) [4,5]. Although these immunoassays are simple and reliable, it should be noted that the blood drug levels obtained depend on the antibody and detection principle for each method. In addition, cross-reactions often induce blood level overestimation as compared with LC/MS/MS. The LC/MS/MS method has high sensitivity and specificity, and is thus valuable for simultaneous measurement of immunosuppressants and their metabolites [6-8]. However, LC/MS/MS requires mastering of instrument operating protocols and has a high initial cost.

Electrochemical analysis has been used in various fields due to its high temporal and spatial resolution and simple measurement procedure. Even for compounds that do not show an electrode response, such as glucose and glutamate, a biosensor with high selectivity can be constructed by immobilizing the corresponding enzyme on the electrode surface. Determination of the substrate concentration from the current can be performed by oxidation or reduction of  $H_2O_2$  or NADH generated by an enzymatic reaction on the electrode [9,10]. However, enzymes, which are biological macromolecules, are expensive and have problems with long-term stability and heterogeneity in production lots. In addition, it is difficult to construct biosensors for molecules such as CSA, which does not undergo enzymatic reactions. Therefore, we have investigated the use of organocatalysts such as 2,2,6,6-tetramethylpiperidine *N*-oxyl (TEMPO) instead of enzymes to determine the substrate concentration based on an electrical signal [11]. TEMPO is a stable free radical. In organic synthesis, it is used in conjunction with suitable re-oxidizing agents for the oxidation of alcohols [12-14]. In electrochemistry, the oxidation of alcohols is catalyzed by the application of an electric potential under basic aqueous conditions or in organic solvents [15, 16]. Since the oxidation current obtained is proportional to the alcohol concentration in the solution, it is possible to quantify alcohols and compounds with hydroxy groups in the molecules (Figure 1) [17]. In this study, we initially evaluated the electrochemical oxidation capacity of alcohols in acetonitrile for various nitroxyl compounds. The ability to oxidize alcohols in organic solvents using an oxidizing agent instead of applying a potential was evaluated and compared with the electrochemical response. Electrochemical detection of CSA, a drug with a hydroxyl group in the molecule, was carried out using the most active NNO.

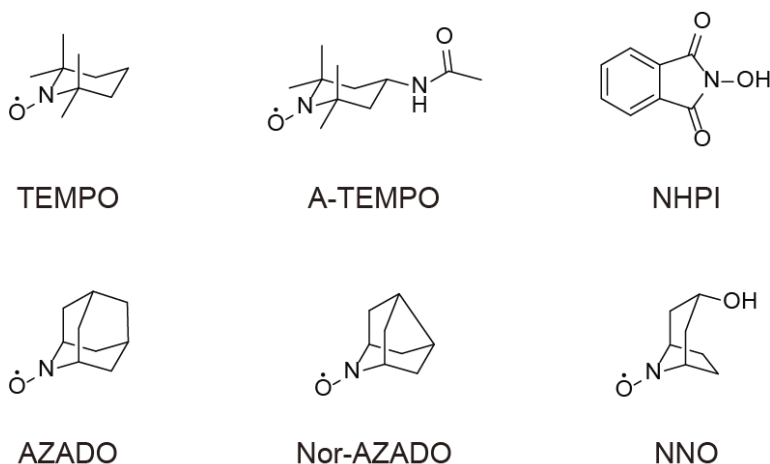


**Figure 1.** Alcohol detection scheme using nitroxyl radical compounds.

## 2. EXPERIMENTAL

### 2.1 Materials

TEMPO, A-TEMPO, AZADOL and NHPI were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). AZADOL is reported to be converted to AZADO by oxidation and has comparable oxidation activity for alcohols [18-20]. NNO [11] and Nor-AZADO [21] were synthesized according to the literature. Cyclosporin A was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). The chemical structures of the nitroxyl radical compounds and NHPI used in this study are shown in Figure 2.



**Figure 2.** Chemical structures of the nitroxyl radical compounds and NHPI used in this study.

### 2.2 Electrochemical Measurements

Cyclic voltammetry (CV) was performed using an electrochemical analyzer (ALS model 660B, BAS, Tokyo, Japan) in a three-electrode cell comprising a Au electrode (diameter: 3 mm) as a working electrode, a platinum wire as a counter electrode, and a Ag/Ag<sup>+</sup> reference electrode. Electrochemical measurements were conducted in acetonitrile containing 100 mM tetrabutylammonium perchlorate

(TBAP). The third CV cycle was recorded. Measurements were carried out several times and the average values were plotted. All experiments were performed at room temperature (ca. 20 °C).

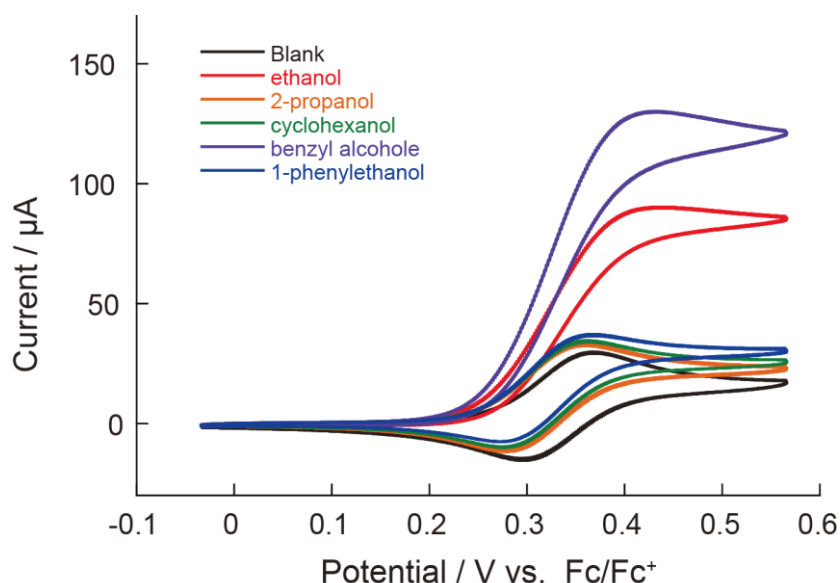
### 2.3 Nitroxyl Radical-catalyzed Alcohol Oxidation with an Organic Oxidant

The catalytic activities of TEMPO, AZADO, and NNO for alcohol oxidation with an organic oxidant were evaluated according to the following reaction procedure. To a solution of an alcohol (3-phenyl-1-propanol or 4-phenyl-2-butanol; 1.0 mmol) and a catalyst (TEMPO, AZADO, or NNO; 10  $\mu$ mol) in dichloromethane (0.50 mL), was added (diacetoxyiodo)benzene (1.1 mmol) at room temperature (ca. 20 °C). The mixture was stirred at the same temperature. Reactions were monitored by gas chromatography (GC) performed on an Agilent 7890A GC system with HP-5 capillary column (0.32 mm  $\times$  30 m, 0.25  $\mu$ m, Agilent Technologies).

## 3. RESULTS AND DISCUSSION

### 3.1 Evaluation of Alcohol Oxidation of Nitroxyl Radical Compounds

TEMPO has poor reactivity due to the four adjacent methyl groups required to stabilize the nitroxyl radicals present in the molecule. Iwabuchi developed AZADO as a nitroxyl radical with reduced bulk [20]. However, few examples of the catalytic performance of AZADO under electrochemical conditions have been reported.



**Figure 3.** Cyclic voltammograms for NNO (1.0 mM) in the absence and presence of 10 mM ethanol, 2-propanol, cyclohexanol, benzyl alcohol and 1-phenylethanol in acetonitrile (20 mM 2,6-lutidine, 100 mM TBAP). The scan rate was 100 mV s<sup>-1</sup>.

Figure 3 shows cyclic voltammograms for NNO in the presence of various alcohols. The oxidation current of +0.4 mV vs. Fc/Fc<sup>+</sup> increased in the presence of alcohols. The alcohol oxidation capacity was evaluated as  $\Delta I_p$ , which is the difference between the oxidation current in the presence and absence of alcohol. Upon applying an electric potential, the nitroxyl radical of NNO is oxidized to become an oxoammonium ion and is thus activated [22, 23]. The oxoammonium ion is reduced by the alcohol in the solution and then oxidized again by the electrode to regenerate the oxoammonium ion [16]. If this cycle is fast enough compared to the CV sweep rate,  $\Delta I_p$  increases [24, 25].

To evaluate the reactivity of various nitroxyl radical compounds, the  $\Delta I_p$  values for 10 mM of different alcohols in acetonitrile are summarized in Table 1. The alcohols used were ethanol, 2-propanol, cyclohexanol, benzyl alcohol, and 1-phenylethanol, with sweep rates of 10, 50 and 100 mV s<sup>-1</sup>. NNO showed the highest reactivity among the measured nitroxyl radical compounds and the highest current response, not only for primary alcohols such as ethanol and benzyl alcohol, but also for secondary alcohols such as 2-propanol. When the scan rate was increased to 100 mV s<sup>-1</sup>, no significant change in the current was observed for NNO. In contrast, no change in the oxidation current for TEMPO, which is widely used in existing methods, was observed for any of the added alcohols in the present study. Therefore, A-TEMPO with an acetamide group at position 4 was used to improve the reactivity of the radicals, and the electro-oxidation proceeded. Since TEMPO has a dimethyl group at the 2 position, it is thought that the oxidation reaction does not progress as smoothly as it should near the reaction point. Therefore, similar measurements were performed using AZADO, which has a bicyclic skeleton with reduced TEMPO bulk. It was found that oxidation proceeded with primary alcohols, but not with secondary alcohols. Nor-AZADO with reduced bulk than AZADO also showed an increased current change. However, like AZADO, oxidation did not occur with secondary alcohols and the oxidation current decreased as the sweep rate was increased. NHPI is known to oxidize alcohols by a different reaction pathway than other nitroxyl radical compounds [26,27]. In NHPI, oxidation proceeded with all alcohols except for ethanol.

The measured  $\Delta I_p$  values for various nitroxyl radical compounds were compared using benzyl alcohol at 50 mV s<sup>-1</sup>. While oxidation did not progress in TEMPO, there was a slight improvement of 3.4  $\mu\text{A}$  in A-TEMPO, 3.9  $\mu\text{A}$  in AZADO, and a fourfold increase to 12.4  $\mu\text{A}$  in Nor-AZADO. The NHPI showed a slight improvement to 5.8  $\mu\text{A}$ . However, NNO showed high reactivity with all alcohols, with a value of 108.8  $\mu\text{A}$  for benzyl alcohol, nearly 30 times higher than that for A-TEMPO. The current change was also observed for secondary alcohols albeit with poor reactivity, and  $\Delta I_p$  values of 4.7, 7.3 and 11.0  $\mu\text{A}$  were measured for 2-propanol, cyclohexanol and 1-phenylethanol, respectively.

AZADO and Nor-AZADO with less-hindered nitroxyl radical moieties exhibited high catalytic activity for alcohol oxidation in organic synthesis [19-21]. Interestingly, under the CV conditions, AZADO showed little anodic current and Nor-AZADO showed a much smaller anodic current than NNO. These results prompted us to compare the catalytic activities of NNO and AZADO for alcohol oxidation with a chemical oxidant. The catalytic activities of NNO, AZADOL, and TEMPO for oxidation of primary and secondary alcohols were compared using (diacetoxyiodo)benzene as the terminal oxidant (Table 2) [28].

**Table 1.** Change in oxidation current ( $\Delta I_p/\mu\text{A}$ ) for alcohols using various nitroxyl radical compounds (1 mM nitroxyl radical and 10 mM alcohol in acetonitrile with 2 equivalents of 2,6-lutidine and 100 mM TBAP).

Alcohol	Scan rate (mVs <sup>-1</sup> )	$\Delta I_p/\mu\text{A}$					
		NNO	AZADO	Nor-AZADO	TEMPO	A-TEMPO	NHPI
Ethanol	10	64.9	1.0	7.6	–	2.9	–
	50	69.0	–	4.7	–	3.6	–
	100	62.2	–	4.3	–	2.4	–
2-Propanol	10	7.0	–	–	–	3.1	1.3
	50	4.7	–	–	–	3.8	1.3
	100	3.8	–	–	–	3.4	1.3
Cyclohexanol	10	10.2	–	–	–	2.9	2.0
	50	7.3	–	–	–	3.5	1.4
	100	6.0	–	–	–	3.2	1.4
Benzyl alcohol	10	94.0	6.0	19.8	–	3.5	9.4
	50	108.8	3.9	12.4	–	3.4	5.8
	100	104.8	4.4	10.2	–	3.5	5.0
1-Phenylethanol	10	15.7	–	–	–	3.2	7.1
	50	11.0	–	–	–	2.7	4.3
	100	8.8	–	–	–	2.1	3.7

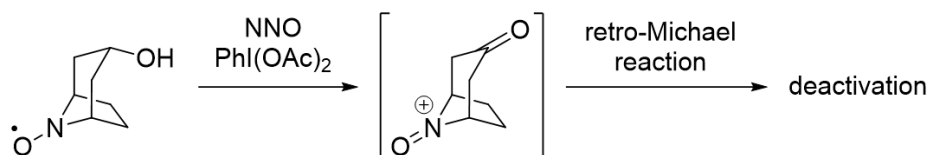
A hyphen means that an electrocatalytic response was not detected.

For the oxidation of 3-phenyl-1-propanol (a primary alcohol), NNO completed the oxidation process within the shortest reaction time (2 hours) (entry 1). AZADO and TEMPO oxidized the same substrate completely within 6 and 9 hours, respectively (entries 2 and 3).

**Table 2.** Comparison of catalytic efficiencies of nitroxyl radicals with an organic oxidant.

Entry	Substrate	Catalyst	Time (h)	GC conv. (%)	Note
1		NNO	2	>99	–
2		AZADOL	6	>99	–
3		TEMPO	9	>99	–
4		NNO	18	71	64% (3 h)
5		AZADOL	4	>99	–
6		TEMPO	18	67	10% (3 h)

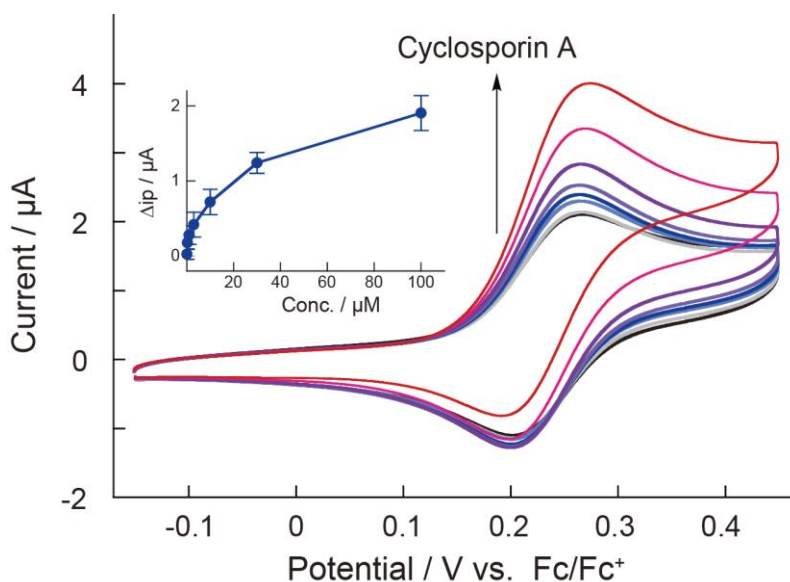
On the other hand, NNO was deactivated within 3 hours in the oxidation of 4-phenyl-2-butanol (a secondary alcohol) (entry 4). AZADO completed the oxidation of the same substrate within 4 hours (entry 5). TEMPO showed a slow reaction rate for the oxidation of the secondary alcohol to afford 67% conversion in 18 hours (entry 6). These results indicate that NNO has higher catalytic activity than AZADO even with a chemical oxidant, but deactivation of NNO could compete in the case of oxidation of a secondary alcohol. The deactivation is most likely due to the oxidation of NNO to a keto-form, which would readily undergo a retro-Michael reaction (Figure 4).



**Figure 4.** A plausible pathway for deactivation of NNO.

### 3.2 Electrochemical Detection of CSA by NNO

The previous evaluation confirmed that NNO has a high oxidation capacity for alcohols in acetonitrile, and the oxidation current suggested the possibility of quantitative analysis. CSA is a cyclic polypeptide antibiotic produced by fungi and has one secondary hydroxyl group in the molecule. However, due to the complexity of the structure, the reactivity is poor and it is difficult to detect using chemical reactions. Nitroxyl radical compounds oxidize hydroxyl groups, and the detected oxidation current can be used to quantify the compounds. However, the conventional reaction with TEMPO does not allow the oxidation of secondary alcohols to proceed. Therefore, we attempted to measure the electrochemistry of CSA using NNO (Figure 5).



**Figure 5.** Cyclic voltammograms for NNO (0.1 mM) in the presence of CSA at a scan rate of 50 mV s<sup>-1</sup>. The inset shows a calibration curve for CSA created from changes in the oxidation current for NNO.

The results showed an increase in oxidation current at CSA concentrations above 1  $\mu\text{M}$ , with a  $\Delta I_p$  of 0.72  $\mu\text{A}$  at 10  $\mu\text{M}$  and 1.90  $\mu\text{A}$  at 100  $\mu\text{M}$ . A plot of  $\Delta I_p$  at +0.4 V vs.  $\text{Fc}/\text{Fc}^+$  against CSA concentration is shown in Figure 5. Since the effective blood concentration of CSA varies depending on the type of disease and on the time of the post-operative course for immunosuppression after organ transplantation, detection in the range of approximately 50-1200 ng mL<sup>-1</sup> (0.042-0.998  $\mu\text{M}$ ) is required [29-30]. The detection range for the calibration curve obtained in the present study was 1-100  $\mu\text{M}$  (1202-

120261 ng mL<sup>-1</sup>), which is a sufficient response. A linear relationship between  $\Delta I_p$  and the concentration of CSA from 1 to 10  $\mu$ M was obtained. On the other hand, the calibration curve in the CSA concentration ranging from 10 to 100  $\mu$ M was not linear. This is because the reaction between NNO and CSA proceeds by catalytic EC (E represents an electron transfer at the electrode surface, and C represents a homogenous chemical reaction) reaction (Figure 1). A homogenous chemical reaction between the oxoammonium ion and CSA is rate-determining at high concentrations of CSA. Table 3 shows a comparison with previously reported methods for detection of CSA. The electrochemical method was low sensitive than the previously reported methods, but it has the advantage of being a simple and inexpensive method that does not require expensive measurement devices.

**Table 3.** Comparison with previously reported methods for detection of CSA.

Method	Detection range (ng mL <sup>-1</sup> )	Sensitivity	Reference
ACMIA	25-500	–	[2]
ECLIA	30-961	–	[5]
LC/MS/MS	4.5-1500	LOD: 0.05 ng/ mL <sup>-1</sup>	[6]
LC/MS/MS	12.5-1825	LLOQ: 12.5 ng mL <sup>-1</sup>	[8]
CV (NNO)	1202-120261	LOD: ca. 360.8 ng mL <sup>-1</sup>	This work

LOD: limit of detection, LLOQ: lower limit of quantification.

#### 4. CONCLUSIONS

The electrochemical oxidation capacity of various nitroxyl radical compounds for alcohols in acetonitrile was evaluated. A high response current was obtained with NNO. The electrochemical measurement of CSA, a TDM drug, was successfully carried out. The detection range was 1-100  $\mu$ M. This performance is sufficient for clinical testing when combined with a rapid pretreatment method.

#### CONFLICTS OF INTEREST

The authors declare no conflict of interest.

#### ACKNOWLEDGEMENTS

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