Effect of Saliva on Measurement of Chemiluminescence by a Micro-Channel Incorporating a Micro-Channel

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Effect of saliva on measurement of chemiluminescence was examined by a micro-reactor incorporating a micro-channel. Sodium hypochlorite and hydrogen peroxide solutions were delivered into a micro-channel developed in a micro-reactor by a syringe pump, providing a laminar flow liquid-liquid interface in the channel and leading to chemiluminescence from singlet oxygen. It was found under certain conditions including saliva that ca. 5% chemiluminescence of the total chemiluminescence was lost in the micro-channel through the reaction of saliva with singlet oxygen or hydrogen peroxide.

Key words: micro-reactor, chemiluminescence, saliva.

1. Introduction

Recently, miniaturized chemical analysis systems, commonly referred to as micro-total analysis systems, have been reported in various fields.1,2 A micro-reactor consisting of a micro-channel has several features useful for chemical reactions; for example, rapid heat exchange and mass transfer that cannot be achieved using a conventional batch-wise system and laminar flow, which can be obtained in a micro-fluidic system.3,4

On the other hand, active oxygen (singlet oxygen, hydroxyl radicals, superoxide radical anions, hydrogen peroxide, and hypochlorite ion) and various antioxidants have attracted a great deal of attention from the viewpoint of not only specific chemical species in chemical reactions but also medical science with regard to disease factors, health maintenance, and aging.5,6

In our previous studies chemiluminescence (CL) from luminol or singlet oxygen was examined in the micro-channel in a micro-reactor; the observed CL in the channel in a micro-reactor provided quite specific CL behavior, compared with that observed in a batch-reactor.7,8 As to the CL from singlet oxygen, the effect of various antioxidants (sodium azide, histidine, nitroblue tetrazolium, and 2-propanol)9 as well as various beverages possibly including some antioxidants (green tea, coffee, wine, and sake (rice wine))10 were examined by a micro-reactor incorporating the micro-channel.

In the present study we examined the effect of saliva on the measurement of CL by a micro-reactor incorporating the micro-channel. Saliva can be easily

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collected as a sample without any specific care and technique. In addition, researchers have recently become interested in saliva as an indicator or the health and disease factors in a living organism.\textsuperscript{13,14} Although the present findings were fragmentary, they provided valuable information regarding the analysis of saliva as an organism, using a micro-reactor incorporating the micro-channel.

2. Experimental

All reagents used were commercially available and of analytical grade. Ion-exchanged water was distilled for use. Sodium hypochlorite, hydrogen peroxide solution (30wt%), and heavy water were purchased from NacalaiTesque (Kyoto, Japan).

Human saliva was collected using Salivette (SARSTEDT; Aktiengesellschaft & Co., Numbrecht, Germany) in accordance with the manufacturer’s instructions. The swab was chewed or placed under the tongue for \textit{ca}. 45 s, then returned to the Salivette case, followed by centrifugation at 2200 rpm for 10 min. The saliva thus obtained (\textit{ca}. 1.5 ml) was diluted in a buffer solution as necessary.

We used an SBT Kit (ZK01-50; Dojindo, Kumamoto, Japan), which uses a colorimetric method developed to determine chloride radical concentration, to examine the concentration of sodium hypochlorite in Reagent 2 in accordance with the manufacturer’s instructions.

Figure 1 shows an illustration of the micro-reactor used in the present study, incorporating a micro-channel (300 \textmu m in width \times 200 \textmu m in depth) made of polymethylmethacrylate.\textsuperscript{6,15} Reagent 1 was 7.5 mM hydrogen peroxide in 100 mM carbonate buffer (pH 10.8, H\textsubscript{2}O/D\textsubscript{2}O=1/1 (v/v)). Reagent 2 was 2.5 mM sodium hypochlorite in 100 mM carbonate buffer (pH 10.8, H\textsubscript{2}O/D\textsubscript{2}O=1/1 (v/v)). When examining the effects of saliva on the CL intensity, Reagent 2 included a given amount of saliva. The two reagent solutions were delivered with a syringe pump (MF-9090; Bioanalytical Systems Inc., West Lafayette, IN, US). They were joined at the junction point and subsequently fed into the micro-channel. The CL was detected at the detection point (10 mm length along each channel). The detection points were named Points 1–9 along the channel from the junction point on the micro-reactor. In this study, Point 2 was used, as described in the figures (Point 2 was recommended for increased sensitivity of CL measurement, based on our previous work\textsuperscript{11}). The resulting CL in the micro-channel was detected with a photomultiplier tube (H5783; Hamamatsu Photonics Co. Ltd., Shizuoka, Japan) located under the micro-reactor, measured with a CL detector (Model EN-21; Kimoto Electric Inc., Osaka, Japan), and treated with an integrator (Chromatopac C-R6A; Shimadzu Co., Kyoto, Japan).

3. Results and Discussion

As described in the experimental section, the sodium hypochlorite and hydrogen peroxide were delivered into the micro-channel to form a laminar flow liquid-liquid interface (the Reynolds number was calculated as \textit{ca}. 18 at 100 \mu l min\textsuperscript{-1}). Such laminar flow forms a liquid-liquid interface instantly in the micro-channel, and then the interface collapse gradually through molecular diffusion with the residence times. The CL from the singlet oxygen was emitted in the course of the collapse of the interface under laminar flow conditions.\textsuperscript{17} The CL intensity was observed continuously and was stable in the micro-channel as long as the reagents were fed into the channel. Stable and constant CL was recorded as CL intensity (data not shown).

When the reagent flow into the micro-channel was turned “on” and “off” by operating the syringe pump controller, CL from singlet oxygen in the micro-channel responded rapidly to the changes as shown in Fig. 2. The rapid response suggests that CL from singlet oxygen that has a short lifetime\textsuperscript{16,17} was generated through the collapse of the laminar flow liquid-liquid interface.

First, quantitative response of CL intensity and chloride radical concentration was confirmed for various sodium hypochloride concentrations as follows. We
**Fig. 1.** An illustration of the micro-reactor containing a micro-channel. Top view.

**Fig. 2.** The influence of saliva in Reagent 2 on CL intensity using the present micro-reactor with CL detection system. Conditions: Reagent 1, 7.5 mM hydrogen peroxide in 100 mM carbonate buffer (pH 10.8, H₂O/D₂O=1/1 (v/v)); Reagent 2, 2.5 mM sodium hypochlorite in 100 mM carbonate buffer (pH 10.8, H₂O/D₂O=1/1 (v/v)) containing saliva diluted 10, 100, and 1000-fold, left to stand for 30 min at room temperature before measurement; flow rate, 100 μl min⁻¹; and detection point, Point 2. “ON” and “OFF” indicate the times when the syringe pumps were turned on and off, respectively.
examined the relationship between the sodium hypochlorite concentration in Reagent 2 and the CL intensity (data not shown); the sodium hypochlorite concentration was directly calculated from the dilution ratio of the commercial reagent with the buffer solution. The CL intensity responded linearly against the sodium hypochlorite concentration over the range of 0.25 – 2.5 mM (the coefficient values of 0.999). We also examined the relationship between sodium hypochlorite concentration in Reagent 2 and the amount of chloride radical concentration obtained using an SBT Kit. A linear relationship was observed between them, with coefficient values of 0.999.

The influence of saliva in Reagent 2 on CL intensity was examined using the present micro-reactor with CL detection system. The CL intensity was measured using Reagent 2 containing saliva diluted 10, 100, and 1000-fold; Reagent 2 containing saliva was left for 30 min at room temperature before measurement. The CL intensities decreased to 70, 85, and 93%, respectively.
compared to that in the absence of saliva as shown in Fig. 2. Furthermore, we found that the CL intensity gradually decreased as standing time increased (0.5 - 24 h) for Reagent 2 containing saliva diluted 10-fold (Fig. 3). Saliva without sodium hypochlorite left standing for 24 h gave no CL change compared to saliva left for 30 min. The data confirm that some components of saliva reacted with the hypochlorite ion, active oxygen, in Reagent 2 to reduce CL intensity.

Based on Fig. 3, each Reagent 2 solution containing saliva (10-fold dilution) was left for a specific time (0.5 - 24 h) and then the chloride radical concentration measured using an SBT Kit. Saliva at various standing times reduced the concentration of chloride radical. By use of the concentrations of chloride radical and the above-mentioned data (the relationship between sodium hypochlorite concentration in Reagent 2 and the amount of chloride radical concentration obtained using SBT Kit), we estimated the concentrations of sodium hypochlorite in Reagent 2. In addition, we were able to estimate the CL intensities (the possible CL intensities) under the conditions described in the figure captions of Fig. 3 by use of the estimated sodium hypochlorite concentrations and the data of the relationship between the sodium hypochlorite concentration in Reagent 2 and the CL intensity mentioned above.

When we compared the possible CL intensities with measured CL intensities in Fig. 3, the measured CL intensities were lower by ca. 5% than the possible CL intensities. Although the components of saliva, such as lactic acid, enzymes, and proteins, are thought to react with active oxygen, such as hypochlorite ion, the above comparison indicated that not all the decrease in CL in Fig. 3 was caused by the reaction of saliva components with the hypochlorite ion in Reagent 2. That is, ca. 5% CL of the total CL was lost in the micro-channel through the reaction of saliva with other active oxygen, singlet oxygen or hydrogen peroxide. The data obtained here provide information for the analysis of saliva as samples from living organisms from the viewpoint of antioxidants, making use of a micro-reactor consisting of a micro-channel.

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