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ORIGINAL PAPER

Development of a highly sensitive Prussian-blue-based enzymatic biosensor for L-carnitine employing the thiol/disulfide exchange reaction

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Abstract
This is the first report of conducting proof-of-concept study for amperometric acetyltransferase-based L-carnitine sensor by employing the thiol/disulfide exchange reaction. The carnitine acetyltransferase (CrAT) catalyzes the reaction between acetyl-CoA and L-carnitine to produce CoA which is difficult to detect directly by electrochemical methods owing to steric hindrance and electrostatic effect of CoA. The thiol/disulfide exchange reaction between CoA and cystamine was mediated in the enzymatic reaction to produce electrochemically detectable low molecular weight of cationic cysteamine. The sensor exhibited high sensitivity and selectivity for L-carnitine in the concentration range 0.28–50 μM with a limit of detection of 0.28 μM. This is a promising strategy for L-carnitine sensing in point-of-care testing applications.

Keywords L-Carnitine sensor · Prussian blue-modified electrode · Thiol/disulfide exchange reaction · Amperometry

Introduction

Carnitine, a quaternary ammonium antioxidant and anti-inflammatory compound derived from amino acids [1, 2], is mainly distributed in the digestive tract and liver. Carnitine exists in the mitochondria in the cells and plays a role in converting long-chain fatty acids such as visceral fat in the body into physiological energy (adenosine tri-phosphate, ATP, for example). In addition, carnitine reacts with acyl compounds, which are a toxin in the body, to produce carnitine esters, which can be excreted from the cells and out of the body in the urine [3]. About 75% of the carnitine in the body comes from the diet, and the remaining 25% is produced as a by-product of lysine and methionine synthesis in the liver and kidneys. A decrease in the amount of carnitine in the body causes the symptoms of carnitine deficiency, which can trigger various diseases. For example, metabolic encephalopathy, lipid storage myopathy, and cardiomyopathy are typical medical conditions [4, 5]. Therefore, the measurement of carnitine in human body fluids is important for the diagnosis and treatment of various medical conditions.

One of the representative and quantitative analytical methods for carnitine is the radioisotope determination method (RI) [6, 7]. However, despite its high sensitivity and reliability, RI requires specialized knowledge and a complicated analytical procedure. In addition, carnitine measurement by RI is not covered by insurance. Another analytical method for carnitine is colorimetric analysis using Ellman's reagent [8–10]. The measurement process is simple, but desktop-size spectrometry is required for quantitative measurement of the change in carnitine-derived absorbance. Recently, an ion-sensitive field-effect transistor (ISFET)-based enzyme sensor has been developed for carnitine measurement [11]. Carnitine acetyltransferase (CrAT) catalyzes the reversible reaction between acetyl co-enzyme A (acetyl-CoA) and L-carnitine to produce acetyl-L-carnitine

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and free CoA [12–14]. CoA is a thiol compound that can cause pH changes in the vicinity of enzymes immobilized on the pH-sensitive gate electrode of ISFET. However, most physiological solutions have a pH-buffering effect, which decreases the sensitivity of pH-sensitive ISFETs.

Previously, our research group developed a CrAT-based electrochemical biosensor using cathodic stripping voltammetry (CSV) for the highly sensitive and selective detection of carnitine [15]. High sensitivity was achieved by pre-concentration of CoA, a product of the enzymatic reaction between carnitine and acetyl-CoA, on a gold electrode for detection by CSV. However, the measurement process included a change in the measurement solution for pre-concentration of CoA to a basic KOH solution, which has the optimum pH for CSV. Since the measurement processes of these analytical methods are complicated with expensive Au electrode needed, it is still challenging to develop a highly sensitive carnitine sensor with a simple process.

In this study, we propose an amperometric CrAT-based electrochemical carnitine sensor. In this sensor, CoA generated by the enzymatic reaction between carnitine and acetyl-CoA is detected in real-time by simply applying a constant voltage to the sensor electrode. The sensor electrode is a carbon electrode modified with Prussian blue (PB, iron(III) hexacyanoferrate(II)), which is known as a candidate redox mediator for the direct oxidation of thiol compounds at relatively low potentials [16]. However, it has been suggested that the sensitivity of thiol compounds such as CoA is extremely low as a result of large steric hindrance and the electrostatic repulsive force between hexacyanoferrate and the negative charge of CoA [17]. In this study, we propose a new reaction scheme in which CoA produced by the enzymatic reaction is converted into a low-molecular-weight thiol by a thiol/disulfide exchange reaction [16–19]. Three kinds of disulfide compounds were selected, based on their sizes and charges, and the magnitudes of the current responses were compared. Finally, the electrochemical characteristics of amperometric carnitine sensing were evaluated using the selected disulfide compounds. In our knowledge, this is the first report to investigate the effect of disulfides on amperometric detection of L-carnitine employing thiol/disulfide exchange reaction.

Experimental

Reagents

2-Aminoethanethiol hydrochloride (cysteamine), acetyl coenzyme A trilithium salt, D-glucose, and creatine monohydrate were purchased from Fujifilm Wako Pure Chemicals. 2-Mercaptoethanol, cystamine dihydrochloride, and L-carnitine hydrochloride were purchased from Tokyo Chemical Industry Co., Ltd. 2-nitro-5-thiobenzoic acid (TNB) was purchased from Biovision. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Nacalai Tesque. Carnitine acetyltransferase from pigeon muscle (CrAT; ≤ 50 units/mg) was purchased from Sigma-Aldrich. The buffer used in all experiments was phosphate-buffered saline (PBS) solution with the composition of 1.5 mM KH_2PO_3, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 136.9 mM NaCl (pH 7.4).

Fabrication of the PB-modified electrode

The electrode pattern, which was composed of an active area (3 × 5 mm) connected to the lead pattern (1 mm width, 30 mm length), was fabricated by thermal deposition of gold through a stencil mask on a polyethylene naphthalate (PEN) film substrate (125 μm in thickness, Teijin DuPont Films). PB carbon paste (C2070424P2, SunChemical) was painted on the active area, followed by baking for 30 min at 60 °C. A fluoropolymer (5 wt%, Teflon AF1600, DuPont) was applied to the lead pattern and dried on a hot plate for 15 min at 30 °C.

Electrochemical measurements

All electrochemical measurements, cyclic voltammetry (CV) and amperometry, were conducted in the PBS measurement solution containing additives, using an electrochemical analyzer (ALS 612E, BAS Inc.). The PB-modified electrode, Pt-coiled wire, and NaCl-saturated Ag/AgCl electrode (RE-1B, BAS Inc.) were used as the working, counter, and reference electrodes, respectively. For CV measurements, the scan rate was set to 20 mV s^{-1}. All amperometry measurements were conducted by applying a constant potential of + 0.2 V at time $t = 0$ until the end of the measurement while stirring the measurement solution at 300 rpm.

Results and discussion

Figure 1 shows the detection scheme of the enzyme-based amperometric carnitine sensor. CrAT catalyzes the reaction between L-carnitine and acetyl-CoA to produce CoA and acetyl-L-carnitine. The CoA produced reduces PB immobilized on the carbon electrode to form Prussian white (PW). Finally, PW is electrochemically oxidized by a carbon-based working electrode. These sequential reactions generate an amperometric response at the working electrode, depending on the concentration of carnitine (Fig. 1a). On the other hand, as described in "Introduction", it is difficult to oxidize CoA directly on the PB-modified electrode because of steric hindrance and the electrostatic repulsive force caused by the negative charge of CoA [17]. Therefore, we investigated the use of a thiol/disulfide exchange reaction [16–19] to convert

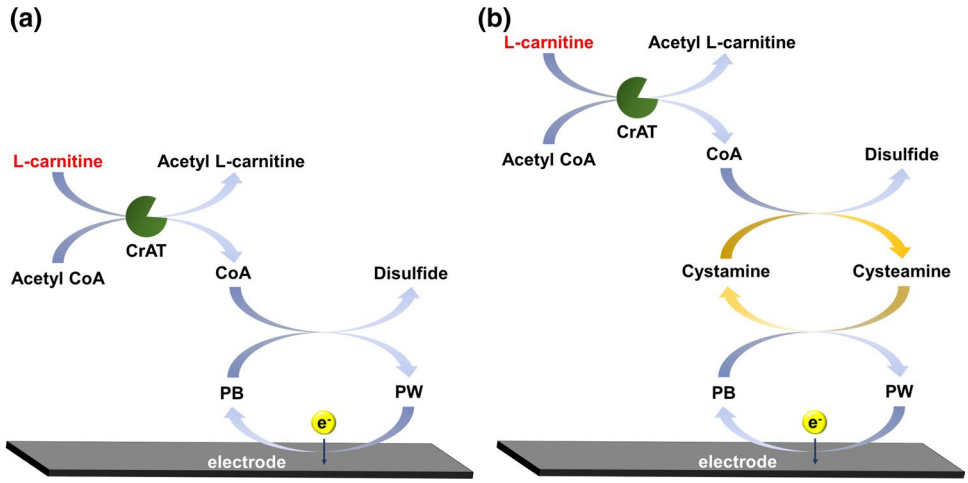

Fig. 1 Schematic view of the enzymatic reaction and electrochemical detection CrAT on the PB-modified electrode. **a**, **b** Enzymatic and electrochemical reaction without (**a**) and via the thiol/disulfide exchange reaction (**b**)

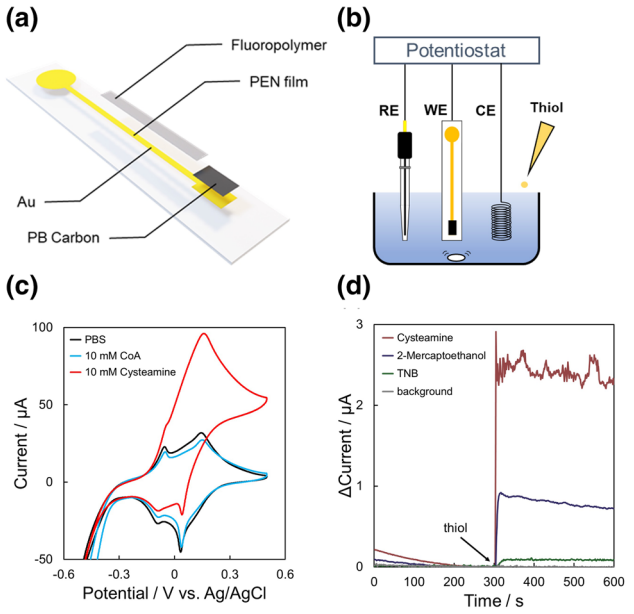

Fig. 2 Experimental setup and the electrochemical characterization of the PB-modified electrode. **a** Structure of the PB-modified carbon working electrode **b** Setup for CV and chronoamperometry using the PB-modified electrode. **c** CVs detected by the PB-modified electrode before and after the addition of 10 mM cysteamine or 10 mM CoA. **d** Chronoamperometric responses for the addition of three kinds of thiols

CoA to a low molecular weight, neutral or cationic thiol compound (Fig. 1b).

First, electrochemical detection of the three thiol compounds was investigated using the PB-modified electrode (Fig. 2a). Figure 2b shows the setup for cyclic voltammetry (CV) and chronoamperometry. These electrochemical measurements were conducted in a three-electrode configuration: the PB-modified electrode, a Pt coil electrode, and a NaCl-saturated Ag/AgCl electrode as the working, counter, and reference electrodes, respectively. The CVs for the PB-modified electrode were detected in phosphate-buffered saline (PBS) solution (pH 7.4) before and after adding a candidate thiol compound. The candidate thiol compounds were cysteamine, 2-mercaptoethanol, and 5-mercapto-2-nitrobenzoic acid (TNB), all of which were chosen as low-molecular-weight and highly reactive cationic thiols. Figure 2c shows the CVs for the PB-modified electrode before (black line) and after the addition of 10 mM CoA (blue line) or 10 mM cysteamine (red line). High concentration of CoA and cysteamine were added to the PBS to show clear difference among CVs. The potential was scanned in the direction from −0.5 to +0.5 V vs. Ag/AgCl. Before the addition of CoA or cysteamine, clear redox peak currents were obtained at +0.15 and +0.05 V vs. Ag/AgCl for the following electrochemical reaction [20–23]:

The CV for CoA in PBS was almost same as that of in PBS, suggesting insensitivity of the PB-modified electrode against CoA. On the other hand, in the CV for cysteamine in PBS, the oxidation current increased while the reduction current decreased. The chemical reaction between PB and cysteamine is as follows:

$$\mathrm{PB} + \text{cysteamine} \rightarrow \mathrm{PW} + \text{cystamine}$$

This chemical reaction increased the amount of PW, resulting in an increase in the oxidation peak current for PW. Decrease in the reduction current for PB indicted the fast reproduction of PW by this chemical reaction, resulting in decreasing the amount of PB. Based on these results, the oxidation potential for PW in chronoamperometry was set to be +0.2 V. Figure 2d shows the chronoamperometric responses for three types of thiols: cysteamine, 2-mercaptoethanol, and TNB. A constant electric potential of +0.2 V was applied at time $t = 0$ s, and the thiols were added at $t = 300$ s to achieve a final concentration of 100 µM. The oxidation currents sharply increased immediately after the addition

of the thiols. The magnitude of the change in the oxidation current increased in the order cysteamine > 2-mercaptoethanol > TNB. The reason for this tendency is probably because cysteamine is a small molecular size and positively charged compound than the other thiols [16].

The thiol/disulfide exchange reaction between CoA and the three-candidate disulfide compounds derived from the thiols studied in Fig. 2 was then evaluated by chronoamperometry, using the setup shown in Fig. 3a. CoA and the disulfide were added to the measurement solution to final concentrations of 100 µM at time = 270 and 300 s, monitoring the amperometric current at + 0.2 V. The following reaction is considered to proceed in PBS:

$$\mathrm{CoA} + \mathrm{R'SSR'} \rightleftarrows \mathrm{CoASSR'} + \mathrm{R'SH}$$

Following this reaction, the three-candidate disulfide compounds, cystamine, bis(2-hydroxyethyl) disulfide, and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), produce cysteamine, 2-mercaptoethanol, and TNB, respectively. R'SH reduces PB at the working electrode, resulting in an increase in the oxidation current for PW. Figure 3b shows the chronoamperometric responses of CoA-reacted cystamine, bis(2-hydroxyethyl) disulfide, and DTNB. The current response increased after the addition of CoA and cystamine. In contrast, almost no current responses were obtained for bis (2-hydroxyethyl) disulfide or DTNB. These results suggest that cystamine, which can produce cysteamine via a chemical reaction with CoA, is a suitable disulfide for the thiol/disulfide exchange reaction-based electrochemical sensor. In the following study, we used cystamine for the electrochemical characterization of an enzyme-based carnitine sensor.

The enzymatic reaction between L-carnitine and acetyl-CoA using CrAT was electrochemically detected via a thiol/disulfide exchange reaction using cystamine. Figure 4a shows the setup for the electrochemical measurement of the enzymatic reaction in the PBS measurement solution. Acetyl CoA (100 µM), CrAT (0.1 units), and cystamine (100 µM) were added to the PBS prior to L-carnitine injection. A constant electric potential of + 0.2 V was applied at time $t = 0$ s, and concentrated L-carnitine was added at 300 s to give final concentrations of 10, 25, or 50 µM. Figure 4b shows the chronoamperometric responses for these concentrations of L-carnitine. The current responses increased in a concentration-dependent manner after the addition of L-carnitine. However, in the absence of cystamine, almost no current response was observed. These results suggest that the thiol/disulfide exchange reaction is effective in amplifying the amperometric current response of the PB-modified enzymatic L-carnitine sensor.

Figure 5 shows the relationship between L-carnitine concentration and current response. Two types of relationships were compared: one is for the rate of current change immediately after the addition of L-carnitine (Fig. 5a, which we call the initial-rate method), and the other is for the change in oxidation current 1500 s after the addition of L-carnitine (Fig. 5b, which we call the endpoint method). In both graphs, the response increased with an increase in L-carnitine concentration up to 50 µM, where it reached a maximum. This is thought to be due to a lack of substrate because the final concentrations of CoA and disulfide were set to 100 µM. Another reason for the decrease in current response at 100 µM is that CrAT catalyzed the reverse reaction of enzymatically produced acetyl-L-carnitine and free CoA as follows:

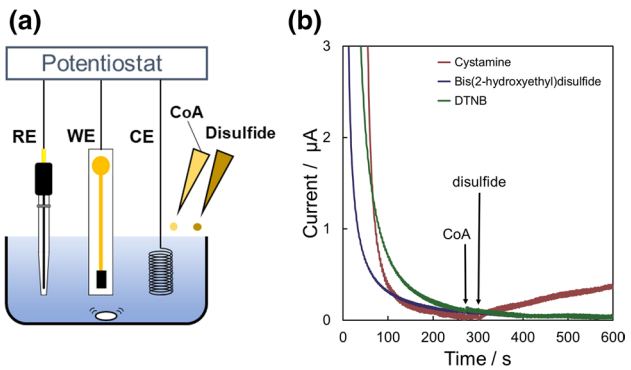

Fig. 3 Comparison of electrochemical responses against three kinds of disulfide compounds. **a** Setup of the electrochemical detection of the thiol/disulfide exchange reaction. **b** Chronoamperometric reactions for the sequential addition of CoA and three kinds of disulfide compounds

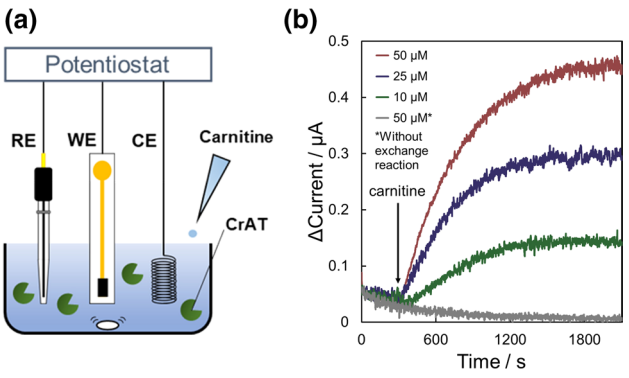

Fig. 4 Electrochemical responses against different concentrations of L-carnitine. **a** Setup for the electrochemical detection of an enzymatic reaction in PBS containing 100 µM acetyl-CoA, 0.1 units of CrAT, and 100 µM cystamine. **b** Chronoamperometric responses of the PB-modified electrode for the addition of different concentrations of L-carnitine in the absence (gray line*) and presence (colored lines) of cystamine

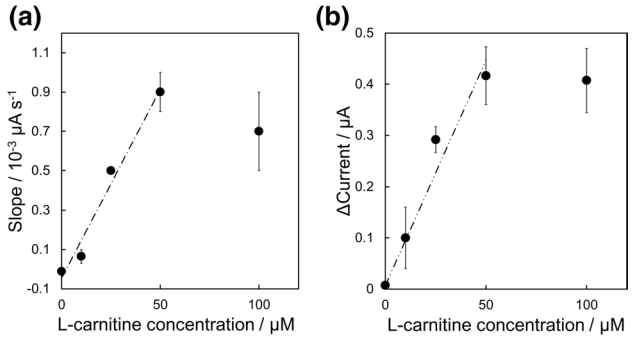

Fig. 5 Calibration curves of the L-carnitine sensor. **a**, **b** Calibration curve of the initial-rate method (**a**) and the endpoint method (**b**)

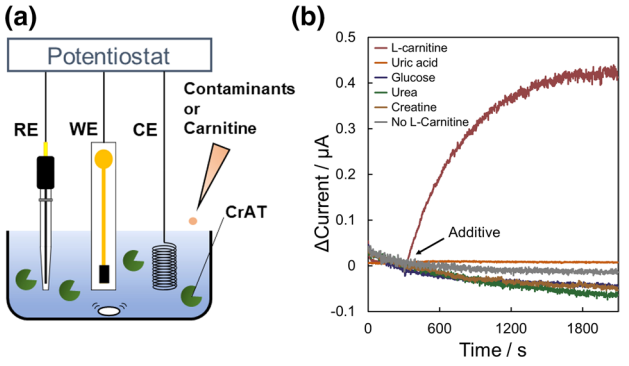

Fig. 6 Evaluation of the sensor selectivity against the typical interferences. **a** Experimental setup. **b** Chronoamperometric responses against L-carnitine and interferences

L-carnitine + Acetyl CoA ⇄ CoA + Acetyl-L-carnitine

As L-carnitine concentration increased, the concentration of enzymatic reaction product (CoA and Acetyl-L-carnitine) increased, followed by occurring the reverse reaction to decrease the current response. The linear range of the calibration curve in Fig. 5a extended from 3.21 to 50 μM, where the linear regression equation was $y = 0.019x - 0.045$ ($R^2 = 0.98$). The linear range of the calibration curve in Fig. 5b was from 0.28 to 50 μM, and the linear regression equation was $y = 0.0088x + 0.0071$ ($R^2 = 0.96$). The limit of detection (LOD) was calculated using the formula LOD = 3.3 × SD/slope, where SD is the standard deviation of the measurements [24], and found to be 3.2 μM from Fig. 5a and 0.28 μM from Fig. 5b. Quantifying L-carnitine using the initial-rate method has the advantage of rapid assay time, but the sensitivity is relatively low, probably because the chemical and enzymatic reactions had not proceeded sufficiently at the initial stage. On the other hand, the endpoint method showed a lower LOD than that of the initial-rate method, probably because the chemical and enzymatic reactions reached sufficient equilibrium, while sacrificing the short assay time. It is necessary to select one of these two calibration curves, depending on the application of the sensor. Note that increasing the concentration of substrates for CrAT (Acetyl CoA and cysteamine) may have possibility to improve the sensitivity and linear range of the calibration curve. Optimizing the concentration of these substrates will be an important issue in the future study.

The selectivity of the L-carnitine sensor was evaluated using the setup shown in Fig. 6a. Acetyl CoA (100 μM), CrAT (0.1 units), and cystamine (100 μM) were added to PBS. A constant electric potential of + 0.2 V was applied at time $t = 0$ s, and the concentrated L-carnitine or four typical interferences in saliva as a model bodily fluid (D-glucose, urea, creatinine, and uric acid, that have been used to assess the selectivity of sensors for saliva [15]) were added at $t = 300$ s to a final concentration of 50 μM. Our previous study has shown that L-carnitine may have possibility to be a biomarker for screening oral cancer risk [25] and therefore we designed this experiment. Previous reports have shown that the concentrations of D-glucose, urea, creatine, and uric acid contained in saliva are 110.17 ± 101.84 μM, 7.56 ± 3.93 μM, 11.45 ± 11.16 μM, 199 ± 27 μM, respectively [26–29]. Almost no current response was obtained for four interferences other than L-carnitine (Fig. 6b). This is thought to reflect the high selectivity of the enzyme toward L-carnitine.

Conclusions

In this study, we developed an amperometric enzyme-based L-carnitine sensor employing a thiol/disulfide exchange reaction. We found that the low-molecular-weight cationic thiol cystamine was suitable for highly sensitive, thiol/disulfide exchange reaction-based amperometric detection of L-carnitine. The detection limit of the present carnitine sensor using the endpoint method was 0.28 μM in the linear range from 0.28 to 50 μM with the help of the thiol/disulfide exchange reaction of cystamine. However, it is still challenging to improve the sensitivity without sacrificing the sensor response speed for point-of-care-testing applications of the present sensor. We believe that electrode surface modification with CrAT enzyme will be a promising strategy for this purpose in future studies.

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Data availability statement All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

References

1. L. Charleston IV, S. Khalil, W.B. Young, Curr. Pain Headache Rep. **25**, 26 (2021)
2. A.W. El-Hattab, F. Scaglia, Mol. Genet. Metab. **116**, 107 (2015)
3. J.L. Flanagan, P.A. Simmons, J. Vehige, M.D.P. Willcox, Q. Garrett, Nutr. Metab. **7**, 30 (2010)
4. G.N. Breningstall, Pediatr. Neurol. **6**, 75 (1990)
5. D.X. Assad, E.C.P. Mascarenhas, C.L. de Lima, I.P. de Toledo, H. Chardin, A. Combes, A.C. Acevedo, E.N.S. Guerra, Int. J. Clin. Oncol. **25**, 1016 (2020)
6. J. Demarquoy, B. Georges, C. Rigault, M.C. Royer, A. Clairet, M. Soty, S. Lekounoungou, F. Le Borgne, Food Chem. **86**, 137 (2004)
7. J.D. McGarry, D.W. Foster, J. Lipid Res. **17**, 277 (1976)
8. C.K. Riener, G. Kada, H.J. Gruber, Anal. Bioanal. Chem. **373**, 266 (2002)
9. T. Ozawa, A. Hanaki, Chem. Pharm. Bull. **29**, 1101 (1981)
10. O. Erel, S. Neselioglu, Clin. Biochem. **47**, 326 (2014)
11. M.S. Andrianova, E.V. Kuznetsov, V.P. Grudtsov, A.E. Kuznetsov, Biosens. Bioelectron. **119**, 48 (2018)
12. N.R. Marquis, I.B. Fritz, J. Lipid Res. **5**, 184 (1964)
13. M.J. Kratochvil, N.K. Balerud, S.J. Schindler, M.A. Moxley, Arch. Biochem. Biophys. **691**, 108507 (2020)
14. D.M. Muoio, R.C. Noland, J.P. Kovalik, S.E. Seiler, M.N. Davies, K.L. DeBalsi, O.R. Ilkayeva, R.D. Stevens, I. Kheterpal, J. Zhang, J.D. Covington, S. Bajpeyi, E. Ravussin, W. Kraus, T.R. Koves, R.L. Mynatt, Cell Metab. **15**, 764 (2012)
15. Z. Zhang, O. Niwa, S. Shiba, S. Tokito, K. Nagamine, S. Ishikawa, M. Sugimoto, Sens. Actuators B: Chemical **321**, 128473 (2020)
16. F. Ricci, F. Arduini, A. Amine, D. Moscone, G. Palleschi, J. Electroanal. Chem. **563**, 229 (2004)
17. P. Nagy, Antioxid. Redox Signal. **18**, 1623 (2013)
18. L.E.S. Netto, M.A. de Oliveira, C.A. Tairum, J.F. da Silva Neto, Free Radic. Res. **50**, 206 (2016)
19. R.E. Hansen, J.R. Winther, Anal. Biochem. **394**, 147 (2009)
20. S. Kongkaew, K. Joonyong, P. Kanatharana, P. Thavarungkul, W. Limbut, Electrochim. Acta **388**, 138558 (2021)
21. D. Rojas, J.F. Hernández-Rodriguez, F.D. Pelle, M.D. Carlo, D. Compagnone, A. Escarpa, Biosens. Bioelectron. **170**, 112669 (2020)
22. C. McBeth, A. Paterson, D. Sharp, J. Electroanal. Chem. **878**, 114537 (2020)
23. A. Chmayssem, N. Verplanck, C.E. Tanase, G. Costa, K. Monsalve-Grijalba, S. Amigues, M. Alias, M. Gougis, V. Mourier, S. Vignoud, A.M. Ghaemmaghami, P. Mailley, Talanta **229**, 122275 (2021)
24. K. Sasaki, H. Furusawa, K. Nagamine, S. Tokito, ACS Omega **5**, 32844 (2020)
25. S. Ishikawa, D.T.W. Wong, M. Sugimoto, F.O. Gleber-Netto, F. Li, M. Tu, Y. Zhang, D. Akin, M. Lino, Clin. Oral Investig. **23**, 3557 (2019)
26. M. Yamaguchi, M. Mitsumori, Y. Kano, I.E.E.E. Eng, Med. Biol. Mag. **17**, 59 (1998)
27. I. Tomás, J.S. Marinho, J. Limeres, M.J. Santos, L. Araújo, P. Diz, Arch. Oral Biol. **53**, 528 (2008)
28. L.D. Martínez, M. Bezard, M. Brunotto, R.D. de Kremer, Acta Odontol. Latinoam. **29**, 49 (2016)
29. A. Vernerová, L.K. Krčmová, B. Melichar, F. Švec, Clin. Chem. Lab. Med. **59**(5), 797 (2021)