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A novel gold nanocluster–based fluorometric biosensor for measuring prooxidant activity with a large Stokes shift

Esin Akyüz, Furkan Burak Şen, Mustafa Bener, Kevser Sözgen Başkan, Reşat Apak*

Department of Chemistry, Faculty of Engineering, Istanbul University–Cerrahpasa, Avcilar, 34320, Istanbul, Turkey

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ABSTRACT

A chicken egg white protein – protected gold nanocluster (CEW – AuNC) based fluorogenic biosensor, where protein was used as both reducing and protecting agent, was developed to determine the Cu(II) – induced prooxidant activity of natural antioxidants abundant in food and biological samples. Gold nanoclusters, prepared using egg white proteins, exhibited strong fluorescence. The prooxidant activity of the tested antioxidants was indirectly measured by their reducing action on Cu(II) to Cu(I), and the reduced cuprous ion was bound to the thiol groups in the CEW – AuNC structure, causing a decrease in fluorescence intensity. Epicatechin, catechin, epigallocatechin gallate, morin, rutin, quercetin, gallic, chlorogenic, and rosmarinic acids, glutathione, cysteine, N – acetyl cysteine, bilirubin, resveratrol, and α-tocopherol were studied as natural antioxidants. A fluorometric method showing a large Stokes shift with excitation/emission maxima at 360/640 nm was developed to sensitively measure the decrease in the fluorescence of CEW – AuNC associated with the binding of copper(I) to the protein structure. Total prooxidant activities of the binary, ternary, and quaternary synthetic mixtures and of some food and synthetic serum samples were determined. The biosensor response was statistically compared to that of its spectrophotometric counterpart. This method can be used for the control of the oxidative stability of foods with a prolonged shelf life.

1. Introduction

Oxygen– and nitrogen–derived free radicals or non–radical reactive species (ROS/RNS) are inevitably produced as a result of the metabolism of aerobic organisms. Reactive species have some critical physiological roles at low levels, in contrast to the fact that they cause oxidative damage in biological macromolecules such as protein, lipid, and nucleic acid when they are excessively present in the organism [1]. Oxidative stress may occur as a result of the imbalance of antioxidants and prooxidants in the presence of copper and iron ions which are major redox–active metals present in serum and tissue. One of the ways to approach prooxidant activity is the capability of antioxidant compounds to reduce redox–active metal ions to their lower oxidation states, stimulating the production of reactive species *via* Fenton–type reactions. The reduced forms of the metal ions may redox–cycle with oxygen species and induce "site–specific" oxidative damage to biological macromolecules [2]. It is important to know which antioxidant at what amount is health–beneficial or may cause oxidative damage in the medium [3]. These compounds can act as prooxidants under certain conditions such as high concentrations, high pH and in the presence of redox–active metal ions [4]. In the last decade, it was mentioned by various researchers that antioxidant compounds may show antioxidative behavior at low concentrations, but act as prooxidants at higher concentrations depending on the medium [5]. In general, antioxidants with stronger reducing ability have a higher chance of displaying structure–dependent prooxidant activity in addition to their dose–dependent prooxidant action. Transition metal ion–induced prooxidant activity also depends on the number of free OH– substitutions on the chemical structure, i.e., the greater the number of phenolic hydroxyl groups, the stronger the prooxidant activity [6].

In the last decades, various gold nanomaterials attracted great attention in different fields of nanotechnology. Especially gold nanoclusters (NCs) below 1 nm in size [7] were used in biological imaging or drug delivery in medicine [8,9], and heavy metal ion detection in environmental and biological systems [10]. In the field of medicine, the use of tiny size AuNCs to reduce toxicity *in vivo* and easy excretion from the body was an important advantage, as opposed to AuNPs that could not be metabolized with their high size in cancer diagnosis and treatment [11]. The green synthesis of stable, water–soluble, and highly fluorescent NCs composed of 25 gold atoms [9] with the use of biological materials as both reducing and the stabilizing agents was a significant superiority [12].

* Corresponding author.
E-mail address: rapak@istanbul.edu.tr (R. Apak).

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The development of cheap, sensitive, easily applicable and repeatable nano–sensing assays which can measure the prooxidant activity of natural antioxidants found in food and biological samples is a priority need in literature, added to the fact that prooxidant biosensor is a rare area in literature, requiring to be filled with more work. Owing to its unclear definition and complicated mechanism, prooxidant activity is a much less visited phenomenon than antioxidant activity subject to a large number of literature assays. In our previous studies, spectrophotometric methods were developed using egg white protein solution [13], calcium proteinate based solid biosensor [14], and chicken egg white protein – protected gold nanocluster (CEW–AuNC) biosensor [15] to measure the Cu(II)–induced prooxidant activity of natural antioxidants.

In this study, a fluorogenic CEW–AuNC biosensor prepared by using proteins acting as both reducing and protecting agents was developed to determine the Cu(II)–induced prooxidant activity of natural antioxidants abundant in plants, foods, and biological samples. Gold nanoclusters were formed using egg white proteins containing excess amounts of thiol groups. The size–tunable strong fluorescence of CEW–AuNCs arises from the molecule–like properties of discrete quantum confined electronic transitions between the HOMO and LUMO (highest occupied and lowest unoccupied molecular orbitals) of atoms within the clusters [16]. In the assay, Cu(II) was reduced to Cu(I) by antioxidants and the reduced form of copper was bound to the thiol groups in the CEW–AuNC structure [17]. A fluorometric method was developed to measure the decrease in the amount of fluorescence of CEW–AuNC in proportion to Cu(I) binding to the protein structure. The proposed fluorometric method manifested itself with a large Stokes shifts (excitation/emission at 360/640 nm). Large Stokes shifts in fluorescence emission make them highly attractive tools for multi–color two–photon *in vivo* imaging [18]. For assays with a high signal–to–noise ratio, a large Stokes shift is desired to minimize cross–talk between excitation and fluorescence emission [18–20]. However, if the used probes have small Stokes shifts in their fluorescence, they are greatly affected by background interferences [19,21]. With the use of 3-mercaptopropionic acid (MPA) as a model ligand and bovine serum albumin (BSA) as a model protein, Deng et al. [22] synthesized water-soluble AuNCs (BSA/MPA-AuNCs) with intense orange-yellow fluorescent emission and a large Stokes shift, which they attributed to thio-Au(I) complexes on the core surface of NCs that display ligand-to-metal charge transfer, ligand-to-metal – metal charge transfer, and ligand-to-metal NP core charge transfer. In general, large Stokes shifts are considered to result from excited-state intra-molecular charge transfer (ICT) between the donor and acceptor, whereas small shifts can cause self-quenching and measurement error by excitation light and scattered light [23]. In ICT, part of the molecule acts as a donor, absorbing light and another portion of the molecule acts as an acceptor, which emits light with a significant red shift. Using this fluorometric sensor, total prooxidant activities of the binary/ternary synthetic mixtures, herbal plant, fruit juice, red wine, and synthetic serum sample were determined, and the obtained results were statistically compared with those of the spectrophotometric CEW–AuNC biosensor.

2. Materials and methods

2.1. Reagents and instrumentation

The following chemicals/reagents were supplied from the indicated sources: resveratrol (RES), catechin (CAT), epicatechin (ECAT), epigallocatechin gallate (EGCG), quercetin (QR), rutin (RT), morin (MOR), chlorogenic acid (CLA), glutathione (GSH), and bilirubin (BLR) from Sigma (Taufkirchen, Germany); gold (III) chloride solution as tetrachloroauric acid ($HAuCl_4$), rosmarinic acid (RA), α–tocopherol (TOC), cysteine (CYS), and neocuproine (2,9–dimethyl–1,10–phenanthroline, Nc) from Aldrich (Taufkirchen, Germany); gallic acid (GA), N–acetyl cysteine (NAC), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$), ethanol (EtOH), copper (II) sulfate, and synthetic serum from Sigma–Aldrich (Taufkirchen, Germany); ethylenediaminetetraacetic acid (EDTA) disodium salt from Fluka (Buchs, Switzerland); disodium hydrogen phosphate (Na_2HPO_4) and sodium hydroxide (NaOH) from Riedel–de Haën (Seelze, Germany). Red wine and apple juice were purchased from a local market. Blueberry and green tea were supplied from a local herbalist.

2.2. Preparation of solutions

Phosphate buffer solution (dihydrogen phosphate-monohydrogen phosphate mixture, pH 7.4, 0.5 M) and $CuSO_4$ solution (2.0 mM) were prepared in pure water. Nc solution (7.5 mM) and the stock solutions of tested antioxidants (10.0 mM) were prepared in EtOH and stored in the refrigerator at −18 °C, except GSH, CYS, NAC, and BLR, which were prepared daily in pure water at the same concentration. The protein and gold nanocluster solutions were prepared as described in our previous work [15].

Red wine, apple juice, and synthetic serum samples were prepared by diluting with distilled water at different ratios. Blueberry and green tea were extracted using the microwave–assisted extraction technique. A 1–g amount of dried sample was transferred to a vessel, 20 mL of 80% aqueous MeOH (v/v) was added, and the rotor with vessels was placed to a microwave oven with stirring. The samples were heated to 80 °C within 3 min, kept at 80 °C for 5 min, and after this period let to cool to room temperature. The extracts were filtered through a GF/PET (glass fiber/polyethylene terephthalate) 1.0/–0.45 μm microfilter before use.

2.3. Preparation of synthetic mixture solutions

Binary, ternary and quaternary combinations of standard antioxidants were prepared for detecting their prooxidant activities in terms of mM ECAT equivalent with respect to the fluorogenic CEW–AuNC based biosensor. The final concentrations of mixture constituents were as follows:

Mixture 1: 14 μM ECAT + 14 μM QR.
Mixture 2: 28 μM CAT + 14 μM MOR.
Mixture 3: 28 μM GA + 14 μM RA.
Mixture 4: 57 μM GSH + 57 μM CYS.
Mixture 5: 14 μM EGCG + 28 μM CLA + 28 μM NAC.
Mixture 6: 14 μM RT + 14 μM RES + 28 μM TOC.
Mixture 7: 14 μM ECAT + 28 μM CLA + 28 μM NAC + 14 μM RES.

2.4. Determination of prooxidant activity using the fluorogenic CEW–AuNC biosensor

To a test tube were added 1 mL of CEW–AuNC, 0.5 mL of phosphate buffer (0.5 M, pH 7.4), 0.5 mL of Cu(II) solution (2.0 mM), (x) mL of standard antioxidant or sample solution, and (1 − x) mL of distilled water. The mixtures were vortexed and incubated for 20 min at room temperature. After this period, 0.5 mL EDTA (0.1 M) solution was added to the mixtures and incubated for 10 min (total volume at 3.5 mL). At the end of this time, fluorescence intensities were recorded (λ_{ex} = 360 nm, λ_{em} = 640 nm). The reagent blank was prepared by adding the same volume of solvent instead of sample solution. The calibration equations were established with the use of the graph plotted between concentration *vs.* the difference in fluorescence intensities of the reference and sample solutions. Total prooxidant activity (TPA) of a synthetic mixture was expressed as mM ECAT equivalent, calculated by summing up the fluorescence differences of sample constituents and dividing the sum by the molar fluorescence coefficient of ECAT (ε_{ECAT}).

2.5. Determination of prooxidant activity using the spectrophotometric CEW–AuNC biosensor

This method was based on measuring the absorbance value of Cu

(I)–Nc chelate formed by neocuproine reaction with protein–bound copper(I) ion which was thought to primarily bind to the thiol groups of the protein in CEW–AuNC structure [15]. After the CEW–AuNC biosensor was incubated with phosphate buffer, Cu(II), and antioxidant solutions for 30 min, EDTA solution was added to eliminate excessive Cu(II) in the medium and vortexed. Neocuproine solution was added immediately to the mixtures (total volume 6.5 mL) to obtain Cu(I)–Nc chelate, and the absorbance value was measured at 450 nm against the blank solution. The reagent blank was prepared by adding the same volume of solvent instead of the standard antioxidant or sample solution.

3. Results and discussion

Li et al. [10] reported that copper and mercury ions quenched AuNC fluorescence emission and sensitive detection of mercury was performed by using EDTA masking method in the presence of equivalent copper ions. In this study, the addition of EDTA stopped the antioxidant–Cu(II) reaction via complexing with the excess Cu(II) in the medium. When Cu(II) is preferentially stabilized (over Cu(I)) by EDTA chelation, Cu(II)–Cu(I) reduction potential decreases (according to the Nernst equation calculating the actual potential in solution), and Cu(II) is no longer able to oxidize flavonoids of which the prooxidant activity would be measured. Thus the redox reaction forming the basis of biosensor action ceases, enabling reproducible measurement. EDTA addition is an important step of the procedure, because the decrease in the intensity of fluorescence caused by protein bound–Cu(I) on CEW–AuNC structure could be precisely determined without interference.

3.1. Optimization of incubation periods

The proposed method comprised two incubation steps, the first (Fig. 1A) of which expressed as the time required for the reduction of Cu(II) to Cu(I), and the second (Fig. 1B) for the complexation of excess Cu(II) with EDTA in the solution phase. The incubation periods were optimized by making triple measurements at different times within the range of 0–60 min using ECAT, GA, and GSH solutions at the same concentration (57 μM). The optimal periods for the first and the second incubations were found as 20 min and 10 min, respectively.

3.2. Analytical figures of merit

The excitation and emission wavelengths of the CEW–AuNC were determined as 360 nm and 640 nm, respectively (see Fig. 2 for fluorescence spectra of the remaining CEW–AuNC in the absence and presence of varying concentrations of ECAT). Thus, the fluorogenic CEW–AuNC based prooxidant biosensor exhibited a large Stokes shifts (280 nm). All the studied antioxidants were checked for the absence of fluorescence at the analytical wavelength (data not shown). In the fluorescent CEW–AuNC biosensor method, the limit of detection (LOD) and limit of quantification (LOQ) values were found to be 0.7 and 2.5 μM ECAT equivalents; the intra–day and inter–day precisions of a 57 μM ECAT solution were 0.4% and 0.8%, respectively.

In the proposed method, calibration equations, correlation coefficients (r), and linear concentration ranges (μM) given in Table 1, were determined from the calibration curves drawn between concentration and the measured intensity difference (ΔI) values. The fluorescence intensity differences were calculated as:

$$\Delta I = I_0 - I$$

where the fluorescence intensities of blank and sample were I_0 and I, respectively.

The order of the molar fluorescence coefficients (ε) of the tested antioxidant compounds for the fluorescent CEW–AuNC biosensor was as follows: BLR > RT > MOR ≥ QR > RA ≥ RES ≥ ECAT ≥ EGCG > CLA > NAC > GA > CAT ≥ GSH ≥ CYS ≥ TOC. As shown in Table 1, a fluorometric method was developed to determine the prooxidant activities of fifteen natural antioxidants belonging to different classes such as flavonoids, phenolic acids, plasma antioxidants, and others. Excellent linearity was obtained between concentration and the difference in fluorescence intensity (Table 1).

Some phenolics, commonly known as strong representatives of antioxidants, may also promote oxidation of other compounds by showing prooxidant activity in some circumstances [24]. Prooxidant activity is known to be induced in the presence of transition metals such as Fe and Cu, especially in biological systems [25]. In addition, parameters such as chelate complex stability, pH and solubility behaviors directly affect the prooxidant activity of compounds [26]. In flavonoids, prooxidant activity is thought to increase in proportion to the total number of hydroxyl groups in their molecules [27]. While the prooxidant activities of mono– and dihydroxy flavonoids cannot be determined, the multiple hydroxyl groups present in the B–ring significantly increase the production of hydroxyl radicals in the Fenton reaction [28,29]. It was reported that 2–3 double bonds and 4–oxo groups present in flavones increase ROS generation induced by Cu (II) in the presence of oxygen [30]. Yang et al. [31] reported the prooxidant activities of QR and *para*–coumaric acid and their derivatives in NADPH/peroxidase/H_2O_2 and DNA cleavage systems. In terms of DNA cleavage activities, QR and RT showed similar prooxidant activity, while NADPH prooxidant activity was found to be RT > QR. In the same study, the number of hydroxyl groups, steric hindrance, concentration and peroxidase affinity were reported to be critical for the prooxidant activities of the tested compounds [31]. EGCG is an effective apoptosis–inducing agent and has bactericidal activity, which is attributed to its ability to reduce O_2 to yield H_2O_2 [32–35]. Oxidative DNA damage due to superoxide anion and hydroxyl radical formation in the presence of EGCG has been reported to be greater than that of ECAT. In addition, it was found that copper oxidized catechins have higher prooxidant activities than non–oxidized forms [36]. Cheng et al. [37] reported the proxidant behavior of phenolic acids such as gallic acid derived from iron redox recycling reactivity. In their study, they investigated the effects of phenolics on the formation of hydroxyl radicals in a Fenton–type system. In addition, Ten et al. [38] reported that the proxidant behavior of GA is due to its strong reducing power and weak metal chelating ability. Summers and Felton evaluated the ability of some phenolic acids to induce oxidative stress in *Helicoverpa zea*. As a result of *in vivo* studies, increased lipid peroxidation products, oxidized protein, and free iron levels showed the prooxidant effect of chlorogenic acid [39]. RA is a well–known prooxidant agent because it can produce reactive oxygen species in the presence of transition metals [40–42]. It was reported that the complex of RA and iron inactivated an aconitase which is the most sensitive enzyme to oxidative stress. In addition, transition metals reduced by RA can form superoxide radical by one electron reduction of oxygen molecule [42]. Frankel et al. reported that RA had low antioxidant activity at 30 ppm but was prooxidant at 50 ppm [43]. Bilirubin, a well known biological antioxidant, has been reported to exhibit prooxidant activity in the presence of a transition metal ion Cu(II) [44–46]. The bilirubin–Cu (II) complex produces free radicals, particularly the hydroxyl radical, and causes strand cleavage in DNA. Prooxidant activities of thiol group antioxidants such as GSH and NAC were reported in literature [47–49]. In the presence of O_2 or trace metal ions or depending on factors such as the nature of the parent thiol compound or local pH, various radicals, including thiyl radicals formed by thiols, can increase the production of hydroxyl radicals produced by the reaction of metals with H_2O_2, resulting in damage to membrane lipids and DNA [49]. Considering the findings in our study, tocopherol had the lowest prooxidant activity among the studied standards. Fukumoto and Mazza [50] confirmed our findings in their study and were unable to detect the prooxidant activity of tocopherol among many antioxidant standards. Antioxidant properties of red wine polyphenols have been reported to be much higher than those of many vitamins (A, C, and E). Since it is known that prooxidant activity and

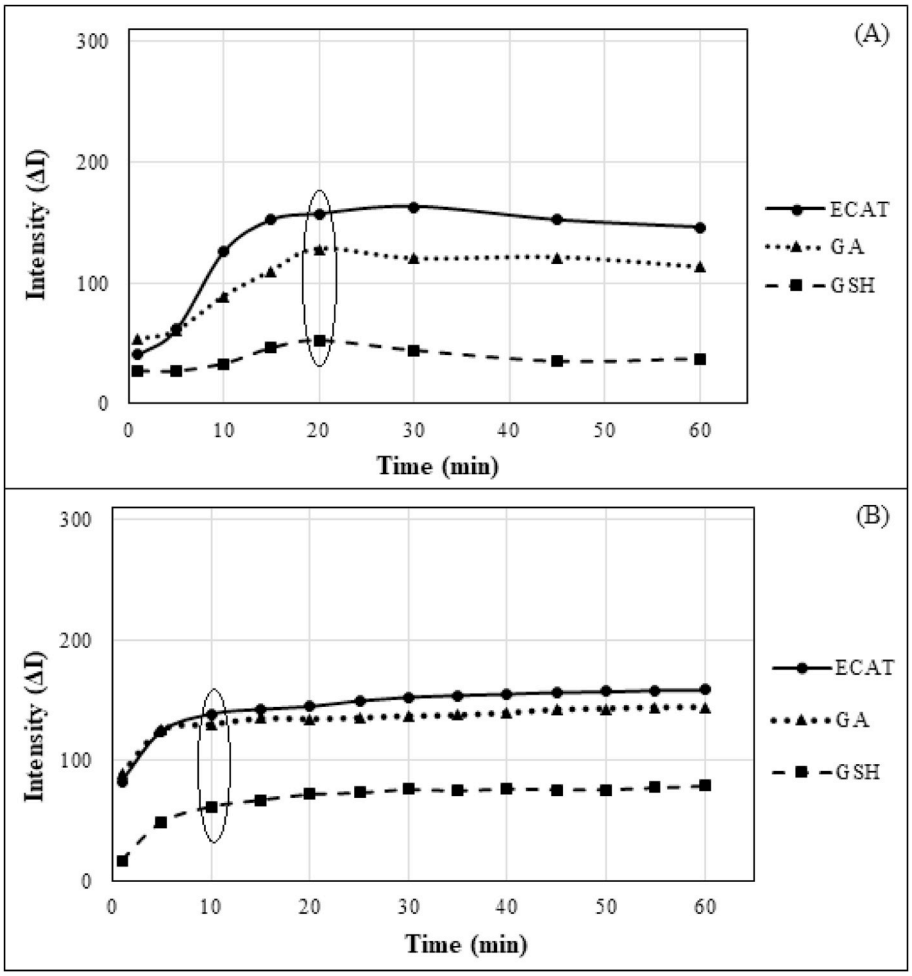

Fig. 1. Incubation period optimization before (A) and after (B) EDTA addition with 57 μM ECAT, GA, and GSH (as the same final concentration) by the proposed assay.

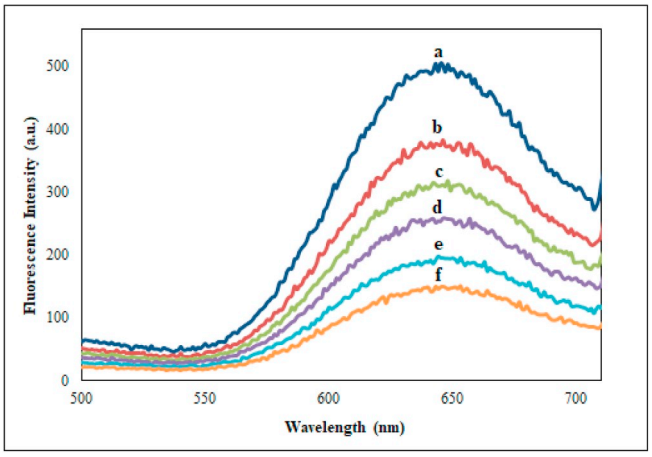

Fig. 2. Fluorescence spectra of the remaining CEW-AuNC in the absence and presence of ECAT (final concentration) (a: 0 μM ECAT (reference), b: 28 μM ECAT, c: 57 μM ECAT, d: 85 μM ECAT, e: 114 μM ECAT, f: 142 μM ECAT).

antioxidant capacity are known to be closely associated with each other, resveratrol supports prooxidant activity stronger than tocopherol as in our study [51].

For the *F*–test comparison of the fluorescent and UV–Vis–based CEW–AuNC biosensors, the TPA values of the ECAT and blueberry extract (5–fold diluted) were calculated as mg ECAT L^{-1} equivalents.

Table 1
Calibration equations, correlation coefficients (r), and linear concentration ranges (μM) of the tested compounds for the fluorescent CEW-AuNC biosensor (n = 3).

Standard	Calibration equation	Correlation coefficient (r)	Linear range (μM)
Flavonoids			
ECAT	$\Delta I = 2.86 \times 10^6\ c + 31$	r = 0.999	14–71
CAT	$\Delta I = 0.69 \times 10^6\ c + 25$	r = 0.999	28–142
EGCG	$\Delta I = 2.64 \times 10^6\ c + 33$	r = 0.996	14–71
QR	$\Delta I = 3.62 \times 10^6\ c + 63$	r = 0.998	14–71
RT	$\Delta I = 4.57 \times 10^6\ c + 47$	r = 0.999	14–71
MOR	$\Delta I = 3.83 \times 10^6\ c + 61$	r = 0.998	14–71
Phenolic acids			
GA	$\Delta I = 1.28 \times 10^6\ c + 43$	r = 0.999	28–142
CLA	$\Delta I = 2.06 \times 10^6\ c + 52$	r = 0.999	28–142
RA	$\Delta I = 3.03 \times 10^6\ c + 50$	r = 0.999	14–71
Plasma antioxidants			
GSH	$\Delta I = 0.60 \times 10^6\ c + 50$	r = 0.998	57–285
CYS	$\Delta I = 0.55 \times 10^6\ c + 51$	r = 0.999	57–285
NAC	$\Delta I = 1.83 \times 10^6\ c + 20$	r = 0.999	28–142
BLR	$\Delta I = 8.83 \times 10^6\ c + 57$	r = 0.998	5–29
TOC	$\Delta I = 0.41 \times 10^6\ c + 28$	r = 0.999	28–286
Other			
RES	$\Delta I = 3.02 \times 10^6\ c + 37$	r = 0.999	14–71

According to the results given in Table 2, there was no significant difference at 95% confidence level between the precision of both methods.

The precision and accuracy of the fluorescent CEW–AuNC biosensor

Table 2
F-test comparison of TPA of ECAT standard and the blueberry extract for The fluorescent and UV–Vis-based CEW-AuNC biosensors.

Sample	Parameter	The fluorescent CEW-AuNC biosensor		The UV–Vis based CEW-AuNC biosensor
ECAT standard	No. of samples	5		5
	Average TPA	142.80[a]		135.92[a]
	Standard deviation	1.12		1.53
	Variance	1.25		2.34
	Degrees of freedom		4	
	$F_{calculated}$		1.88	
	$F_{critical}$		6.39	
Blueberry extract	No. of samples	5		5
	Average TPA	67.07[a]		86.29[a]
	Standard deviation	2.28		3.02
	Variance	5.20		9.12
	Degrees of freedom		4	
	$F_{calculated}$		1.75	
	$F_{critical}$		6.39	

[a] (mg ECAT L^{-1} equiv).

Table 3
The precision and accuracy of the fluorescent CEW-AuNC biosensor (N = 3).

Herbal plant	Added (μM)[a]	Expected (μM)[a]	Found (μM)[a]	Recovery (%)[a]	R.S.D. (%)
RES addition to red wine	14.28	30.17	30.13 ± 0.56	99.87	1.86
	28.57	44.45	44.04 ± 1.73	99.08	3.93
GSH addition to synthetic serum	57.14	78.81	76.67 ± 1.80	97.28	2.35
	114.28	135.95	138.33 ± 1.18	101.75	0.85
RA addition to blueberry extract	14.28	25.12	26.05 ± 0.32	103.70	1.23
	28.57	39.41	39.89 ± 0.65	101.22	1.63
CAT addition to green tea extract	28.57	172.05	172.46 ± 1.90	100.24	1.10
	57.14	200.62	200.00 ± 2.61	99.69	1.30
QR addition to apple juice	7.14	11.89	12.37 ± 0.13	104.04	1.05
	14.28	19.03	18.75 ± 0.18	98.53	0.96

[a] Added, expected, found and recovery were calculated on the basis of concentration of added compound.

Table 4
Comparison of experimental and theoretical TPAs of binary, ternary and quaternary synthetic mixtures as mM ECAT equivalent (n = 3).

Mixture	FL based CEW-AuNC biosensor	
	experimental TPA	theoretical TPA
1	1.63 ± 0.02	1.68
2	1.20 ± 0.02	1.18
3	1.29 ± 0.02	1.33
4	1.25 ± 0.01	1.28
5	1.95 ± 0.02	1.93
6	1.57 ± 0.03	1.67
7	2.06 ± 0.02	2.05

were evaluated by spiking the diluted food and biological samples with two different concentrations of the standard antioxidant solution which was the major component of each sample. The results were given in terms of added, expected, and found individual prooxidant activity as μM equivalents of each added compound in Table 3. The obtained recovery (REC%) and relative standard deviation (RSD%) values were between 97.3–104.0% and 0.85–3.93%, respectively.

In this assay, it is possible to maintain linearity of fluorescence signals with concentration, to ensure additivity of total signal due to the linear superposition of a fixed number of components, and to achieve excellent recoveries for spiked analytes to real samples by the standard addition technique [52]. All these favorable properties (i.e. linearity, additivity and quantitative spike recovery) for the utilized biosensor arise from the fact that the strong fluorophore of egg white protein-AuNCs enables quantitative determination with a linear correlation of corrected fluorescence intensity to analyte concentration owing to the fact that significant inner filter effects causing deviations from linearity can be avoided by keeping the analyte concentrations sufficiently low [52,53]. The strong fluorescence of this single fluorophore (of CE-W—AuNCs) is drastically attenuated by antioxidants causing copper(I) binding to fluorophore amino acids (especially thiols) of AuNC-bound proteins in dilute solutions, enabling a sensitive determination of prooxidant activity in a reasonably linear concentration range.

3.3. TPAs of synthetic mixtures

TPAs of the binary, ternary and quaternary synthetic mixtures were determined in terms of mM ECAT and compared with those theoretically found. The theoretical TPAs of synthetic mixtures were calculated as:

TPA of synth. mix. = $\{(\Delta I_1 + \Delta I_2 + \ldots + \Delta I_n)/\varepsilon_{ECAT}\} \times$ (total vol./sample vol.) × 1000.

The experimental and theoretical TPAs given in Table 4 were highly compatible, and had a strong correlation with each other (r = 0.992).

3.4. TPAs of food and biological samples

Green tea (*Camellia sinensis*), red wine, dried blueberry (*Vaccinium myrtillus*), apple juice, and synthetic serum were studied as real samples. In literature, the main antioxidant components were as follows: in blueberry [54], phenolic acids, anthocyanins, flavanols, and flavonols; in apple [54], chalcones, flavanols, flavonols, and condensed tannins; in green tea,[50] flavanols, flavonols, and phenolic acids; in red wine [51,54–56], anthocyanins, flavanols, flavonols, and especially resveratrol; in biological fluids [45], bilirubin and some aminothiols. The fluorescent and UV–Vis–based CEW–AuNC biosensors were applied to microwave–assisted green tea, blueberry extracts, and directly diluted red wine, apple juice, and serum samples. The TPA values of food and biological samples obtained by both methods, shown in Fig. 3, were expressed as mM ECAT equivalent and were compatible with each other.

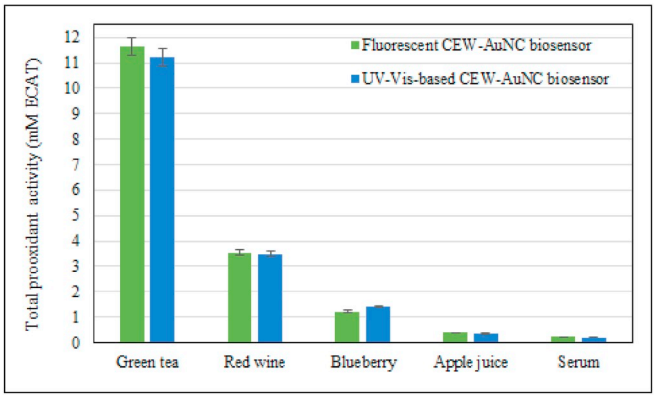

Fig. 3. Comparison of TPAs of green tea and blueberry extracts, red wine, apple juice and synthetic serum for the fluorescent and UV–Vis-based CEW-AuNC biosensors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Conclusion

The prooxidant activity measurement of food and biological samples with the use of AuNCs is a relatively new research area. Although the method procedure was described in the solution phase, the reaction actually occurred on the CEW–AuNC biosensor structure. By the principle of green chemistry, the total solution volume required for the analysis was reduced to 3.5 mL, and the test procedure was completed within 30 min. The limit of detection values of both spectrophotometric and fluorometric CEW–AuNC sensing methods were close to each other, though with a slightly lower detection limit for the current method. As there is currently no simple spectroscopic procedure for TPA measurement, the proposed assay was easily applied to the wide range of fifteen standard antioxidants such as flavonoids, phenolic acids, plasma antioxidants, and others. In the developed method, the prooxidant activities of binary, ternary and quaternary synthetic mixtures were determined with high accuracy, although the additivity was low in most prooxidant activity assays in literature. The CEW–AuNC biosensor based on fluorescence measurement with a large Stokes shift was an easily applicable method for determining prooxidant activities of natural antioxidants commonly found in food and biological samples. The prooxidant activities of the tested compounds were in parallel with the antioxidant capacity values obtained by widely used antioxidant determination methods (CUPRAC, ABTS, DPPH, and Folin) based on electron transfer (ET). As a common feature, the proposed prooxidant biosensor and ET-based antioxidant determination methods rely on the reducing power of the antioxidant compounds. The proposed sensitive and practical sensing method may make a significant contribution to the control of oxidative stability of foods with a prolonged shelf life.

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