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Protein-Protected Gold Nanocluster-Based Biosensor for Determining the Prooxidant Activity of Natural Antioxidant Compounds

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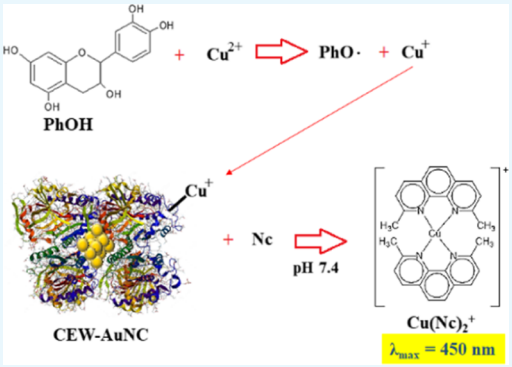
ABSTRACT: In this work, chicken egg white protein (CEW)-protected gold nanoclusters (CEW-AuNCs) were prepared from CEW and $HAuCl_4$ to measure the Cu(II)-induced prooxidant activity of antioxidant compounds such as epicatechin, epigallocatechin gallate, catechin, rosmarinic acid, resveratrol, ascorbic acid, and glutathione. These compounds reduced Cu(II) to Cu(I), and the latter was mainly bound to thiol groups in the CEW-AuNC structure. As the protein-bound Cu(I) may act as a catalytic center for generating reactive oxygen species, the Cu(II) reducing ability of antioxidants is an indirect measure of their prooxidant potency. The bound Cu(I) may be released with the cuprous-selective ligand neocuproine (Nc), forming the basis of a spectrophotometric method measuring absorbance at 450 nm wavelength of the Cu(I)−Nc chelate. The developed method involved a one-pot synthesis and determination without preseparation and was applied to binary synthetic mixtures of studied antioxidant compounds and to certain herbal plant (green tea, linden, echinacea, and artichoke leaf) extracts to determine the total prooxidant activities. The obtained results were statistically compared with those of the literature Cu(II)−Nc assay using a calcium proteinate-based solid biosensor. The developed biosensor was durable, reliable, easily applicable, and of low cost and wide linear range and could determine the prooxidant activities of natural antioxidant samples with high reproducibility.



1. INTRODUCTION

In living organisms, reactive oxygen species (ROS), reactive nitrogen species (RNS), and free radicals are unavoidably formed during normal cellular metabolism. Oxidative stress occurs when prooxidants/oxidants dominate over antioxidants in an impaired balance; this condition causes damage to the biological macromolecules in the organisms leading to various diseases.[1] Intrinsic and extrinsic antioxidant defenses of the organisms combat against ROS/RNS. Although the main health-beneficial effects of natural bioactive compounds originate from their antioxidant properties, they may exhibit prooxidant behavior under certain conditions (such as transition-metal ions and O_2).[2] One possible definition of prooxidant activity is the ability of reducing transition-metal ions to their lower oxidation states by antioxidant compounds, stimulating the production of reactive species which can cause various diseases via Fenton-type reactions.[3] Therefore, understanding the antioxidant/prooxidant behavior of bioactive substances depending on the structures and conditions in which they are found is of great importance.

Iron and copper ions are essential for electron-transfer reactions in biological systems, rendering their dietary intake necessary for all living organisms. However, when these ions are in their free unbound form, they can interact with oxygen by catalyzing Haber−Weiss and/or Fenton reactions. Such reactions give rise to the generation of ROS, which may lead to oxidative damage to biological macromolecules (DNA, proteins, and lipids).[4] These damages may cause many serious diseases such as cardiovascular diseases, neurodegenerative diseases, some types of cancer, and aging.[4−7] The mechanism of oxidative damage by copper(II) in the presence of H_2O_2 probably involves the formation of a copper-coordinated peroxy species or singlet oxygen ($^1O_2$). Cupric ions may further react with superoxide anions to produce hydrogen peroxide and cuprous ions. The indicated redox cycling of Cu(II,I) may catalytically generate hydroxyl radicals in vivo.[5,8,9] In this respect, a full grasp of Cu-induced damage on DNA can be of vital importance in understanding the mechanism of copper-related diseases.

Polyphenolic compounds found in plants and plant foods are known for their antioxidant properties. Fruit- and vegetable-rich diets may give rise to increased polyphenol

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concentrations in the plasma of concerned people, ranging between 0.3 and 10 μM. If polyphenol is oxidized to a corresponding quinone, the involved redox cycling can make that compound a prooxidant. Thus, certain polyphenols owe their prooxidant activity to the redox cycling in which polyphenol-reduced cupric ions (i.e., to the cuprous state) increase the availability of copper to enable the reaction with hydrogen peroxide or other ROS.[5,10]

All kinds of gold nanomaterials [comprising nanoparticles (NPs), nanoclusters (NCs), nanosheets] have been used in diverse scientific and technological fields because of their excellent optoelectrical, chemical, and catalytic properties.[11] Especially, gold NCs (AuNCs) may be distinguished for their facile synthesis, good solubility and fluorescence, photostability, biocompatibility, and reduced toxicity.[12] As opposed to many toxic and environmentally unfriendly organic substances frequently used as reducing or protection agents for the synthesis of noble metal NPs, various biological molecules have recently been used in fluorescent NP/NC synthesis, aiming to overcome biocompatibility-related problems.[13] The use of AuNPs in the field of medicine, especially in cancer diagnosis and treatment, has made great progress. An important advantage of AuNCs is their ability to penetrate to the kidney tissue because of their very small size and to easily dissipate from the body to reduce toxicity in vivo.[14]

Actually, there is a limited number of literature methods for measuring the prooxidant activity of phenolic compounds, some exploiting protein damage.[15−20] Methods aiming at prooxidant activity measurement are distinctively scarce when compared to those focusing on antioxidant activity measurement. As current methods are expensive, laborious, and limited with respect to application area and interferences, there is a need to develop easily applicable, low-cost, convenient, fast, sensitive, and highly reproducible methods that can determine the prooxidant activities of natural antioxidants. In our previous studies, spectrophotometric methods were developed using an egg white protein solution[21] and a calcium proteinate-based solid biosensor[22] to determine the Cu(II)-catalyzed prooxidant activity of phenolic compounds and ascorbic acid (AA) in order to fill the gap in the literature.

In this study, chicken egg white protein-protected gold NCs (CEW-AuNCs) were prepared to measure the transition-metal ion {Cu(II)}-catalyzed prooxidant activities of antioxidant compounds, and accordingly, a spectrophotometric method was developed using reduction of Cu(II) → Cu(I) by antioxidants, uptake of Cu(I) by CEW-AuNC, followed by desorption of Cu(I) from the sensor matrix with the aid of neocuproine (Nc) to produce the chromophore: Cu(I)−Nc chelate having an yellow-orange color and strong absorbance at 450 nm. The developed method was applied to the binary synthetic mixtures of the studied compounds and to herbal plant extracts for determining total prooxidant activities (TPAs). The obtained results were statistically compared with those of the Cu(II)−Nc assay using a calcium proteinate solid biosensor.[22]

2. RESULTS AND DISCUSSION

In the literature, AuNCs were often used in metal ion [such as Fe(III), Cu(II), and Hg(II)] determination[23,24] or biotechnological imaging fields.[13,25] In our study, modified AuNCs were used as a prooxidant sensor for the first time to measure the Cu(II)-catalyzed prooxidant activities of antioxidant compounds. A novel spectrophotometric method based on the absorbance measurement of the Cu(I)−Nc chelate complex was devised, and the Cu(II)-catalyzed prooxidant activities of phenolic compounds such as epicatechin (ECAT), catechin (CAT), epigallocatechin gallate (EGCG), rosmarinic acid (RA), and resveratrol (RES) and other antioxidants such as AA and L-glutathione (GSH) were measured with this sensor. The protein-protected gold NCs (CEW-AuNCs) were formed using CEWs containing thiol groups and gold(III) solution ($HAuCl_4$). In the developed method, the antioxidant compounds reduced Cu(II) to Cu(I), and cuprous ions were assumed to bind mainly to the thiol groups in the CEW-AuNC structure and potentially induce ROS formation. It was proposed that Cu(II) adsorbed to algae was reduced to Cu(I) by −SH groups and bound as a Cu(I)−S-complex and that copper toxicity may principally be due to copper binding to intracellular thiols.[26] Although the standard reduction potential for most semiquinone radical−phenol redox couples is higher than that of [Cu(II)/Cu(I)], that is, 0.17 V, Cu(II) may oxidize some antioxidant phenols (i.e., used in prooxidant activity testing) in the presence of protein thiols and get reduced to Cu(I) because of the relative stability of the Cu(I)−thiol bond raising the cupric/cuprous conditional redox potential. The bound Cu(I) may also exhibit superoxide dismutase activity, leading to the formation of more H_2O_2, and also catalyze the decomposition of H_2O_2 to produce hydroxyl radicals.[27] Copper−protein thiol complexes were proposed as catalytic centers inducing the formation of ROS associated with oxidative damage observed in both Alzheimer's and prion disease.[28] The NC-bound Cu(I) could be finally extracted with an ethanolic Nc solution in the form of a stable $Cu(Nc)_2^+$ complex because of the high stability of the cuprous−Nc chelate in preference to that of Cu(I)−protein thiol bonds. Thus, we propose in this study that copper-catalyzed prooxidant activities of phenolic compounds could be indirectly determined by measuring the absorbance at 450 nm, that is, maximal absorption wavelength of Cu(I)−Nc.

2.1. Optimization of Ethylenediaminetetraacetic Acid Concentration. As the proposed CEW-AuNC assay was performed in a solution medium without separating the NCs, ethylenediaminetetraacetic acid (EDTA) was added to mask the excess Cu(II), which would otherwise easily oxidize any unreacted antioxidant when Nc was added (i.e., Cu(II)—Nc is a strong oxidant having a redox potential of 0.6 V, but in the presence of EDTA, the Cu(II)/Cu(I) reduction potential would be reduced to such a level that excessive Cu(II)—in the chelated form—could not oxidize the remaining antioxidant). In that case, the prooxidant activity could not be precisely measured because of the interference of unmasked Cu(II) causing a positive error. On the other hand, by chelating excessive Cu(II) with EDTA, the added Nc would only react with the already reduced Cu(I) by the test compounds, indicative of their prooxidant activity. In this respect, the interference effects that might arise from the solution medium were eliminated by adding EDTA.

For the optimization of EDTA concentration, EDTA solution was added to the blank at different concentrations (1−100 mM) to decrease the absorbance of the blank. The absorbance measurements of the blank solution with or without EDTA were performed against distilled water. As shown in Figure 1, 0.1 M EDTA with the lowest blank absorbance value was selected. This concentration of EDTA was later used for both the blank and sample solutions.

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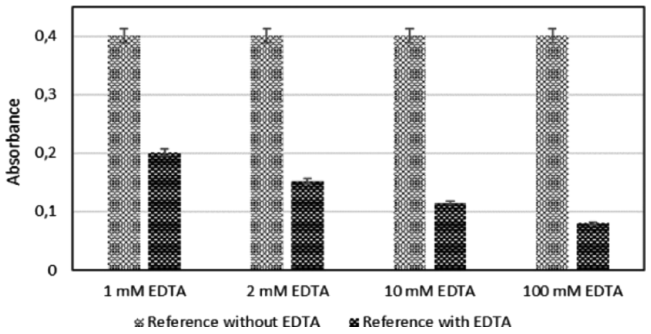

Figure 1. Optimization of the EDTA concentration.

2.2. Optimization of Incubation Periods. The first incubation period (Figure 2A) mentioned in the procedure

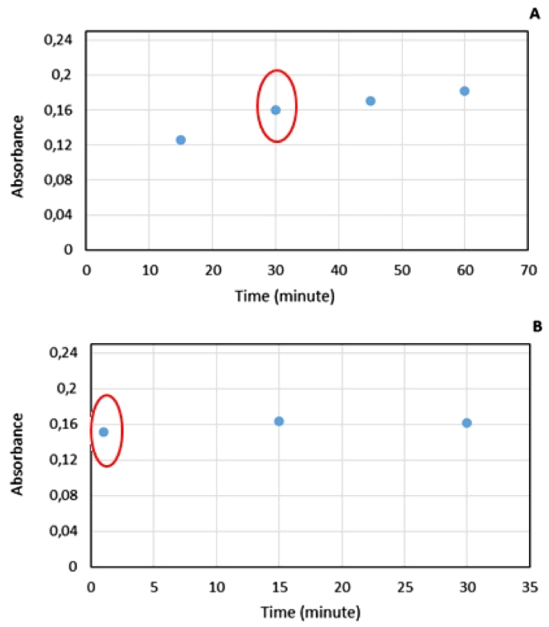

Figure 2. Optimization of the first (A) and second (B) incubation periods for 77 μM ECAT.

was the total time required for the reduction of Cu(II) to Cu(I) in CEW-AuNCs and antioxidant mixtures together with the successive binding of Cu(I) to protein. The second incubation period (Figure 2B) was the time required to break the protein thiol−Cu(I) bond by an Nc solution and to separate the bound copper as a Cu(I)−Nc chelate. The first and second incubation periods were optimized by using 77 μM ECAT solution. For optimization of incubation periods, measurements were taken at different times within the range of 0−60 and 0−30 min.

As shown in Figure 2A,B, the optimal time periods for the first and second incubations were 30 and 1 min, respectively. The first incubation needed longer time because of the completion of a series of reactions comprising a redox reaction (i.e., Cu(II) → Cu(I) reduction) followed by covalent binding of Cu(I) to protein thiols, but the second incubation was fast because of an easy ligand exchange (i.e., Nc binds to protein-bound Cu(I) to produce a distinctively more stable chelate, Cu(I)−Nc) because the conditional stability constants of Cu(I) complexes formed with thiol (log K) and Nc (log β2) ligands were close to 12 and 19, respectively.[29−31] As shown in Figure 2B, waiting for the second incubation was not actually needed as the reaction was almost complete immediately. Therefore, absorbances could be measured rapidly after adding an Nc reagent and vortexing the mixture.

2.3. Analytical Performances of Applied Methods. Limit of detection (LOD) and limit of quantification (LOQ) as well as intra- and interday reproducibility (i.e., within- and between-run precision) of the proposed method as analytical performance for the chosen standard compound (ECAT) are given in Table 1. The correlation coefficients (r) and dynamic

Table 1. Comparison of Figures of Merit of the Biosensors Based on Soluble CEW-AuNCs and a Solid Protein Tested on the ECAT Standard (Number of Measurements: N = 3)

parameter	CEW-AuNC-based Cu(II)−Nc assay	solid protein-based Cu(II)−Nc assay
linear range (μM)	15.4−77	12.5−150
LOD (μM)[a]	0.9	1.2
LOQ (μM)[b]	3.0	4.0
linear equation[c]	$A = 1809c + 0.012$	$A = 3472c + 0.142$
correlation coefficient (r)	0.9977	0.9907
within-run precision[d]	1.3	2.8
between-run precision[d]	3.4	3.2

[a]LOD = $3s_{bl}/m$ (where m is the slope of the calibration line and s_{bl} is the standard deviation of the blank). [b]LOQ = $10s_{bl}/m$. [c]Linear equation between absorbance (A) and concentration (c, M). [d]RSD % for 0.25 mM ECAT.

linear ranges (μM) for the tested antioxidant compounds (ECAT, CAT, EGCG, AA, RA, RES, and GSH) with respect to the Cu(II)−Nc assays using both soluble CEW-AuNC and solid calcium proteinate-based biosensors are shown in Table 2.

The order of the molar absorption coefficients of the tested compounds with respect to the CEW-AuNC and solid protein-based Cu(II)−Nc assays were EGCG > ECAT > CAT > RES > GSH > AA ≥ RA, and EGCG > ECAT > CAT > GSH > AA, respectively. The molar absorption coefficients of antioxidants followed the same order for the two assays, except for the compounds which could not be detected with the reference method. There was a reasonable agreement between the molar absorption coefficients found by the proposed sensing method and those of the solid protein-based Cu(II)−Nc assay, with a correlation coefficient of r = 0.9327 (Table 2). This is a strong positive correlation (N = 5 and r^2 = 0.8699) at $p < 0.05$ level (Spearman rank correlation test), showing that the result is highly significant.

It has been well established in the literature that depending on the reaction conditions, some phenolics may exhibit prooxidant and therefore cytotoxic properties. These parameters affecting the prooxidant ability may comprise transition-metal ion reduction ability, chelate complex stability, hydrogen ion activity (pH), and solubility behaviour.[32] As a leading characteristic, the transition-metal reduction potency of polyphenols may assume the primary role for ROS generation through O_2 reduction by metal catalysis.[33] In a study, 13 polyphenolic compounds were tested to inhibit copper-mediated DNA damage using gel electrophoresis, revealing that both antioxidant and prooxidant abilities depended on polyphenol concentration. In the mentioned study, the

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Table 2. Linear Regression Equations, Correlation Coefficients (r), and Dynamic Linear Concentration Ranges of the Tested Compounds with Respect to the Cu(II)−Nc Assays Applied on the CEW-AuNC Sensor and Solid Ca-Proteinate Sensor (N = 3)[a]

	CEW-AuNC-based Cu(II)−Nc assay		solid protein-based Cu(II)−Nc assay	
tested compound	linear regression equation, and correlation coefficient (r)	linear range (μm)	linear regression equation, correlation coefficient (r)	linear range (μm)
ECAT	A = 1809c + 0.012, r = 0.9977	15.4−77.0	A = 3472c + 0.142, r = 0.9907	12.5−150.0
CAT	A = 1424c + 0.018, r = 0.9951	15.4−77.0	A = 2295c + 0.201, r = 0.9996	25−250
EGCG	A = 3351c + 0.019, r = 0.9993	7.7−76.9	A = 16 354c − 0002, r = 0.9928	6.3−31.3
AA	A = 122c + 0.006, r = 0.9921	154−615	A = 142c + 0.068, r = 0.9998	250−2500
RA	A = 115c + 0.035, r = 0.9974	308−1538	n.d.	n.d.
RES	A = 780c + 0.041, r = 0.9983	61.5−307.7	n.d.	n.d.
GSH	A = 732c − 0.115, r = 0.9945	308−1538	A = 1092c − 0.010, r = 0.9875	50−125

[a]n.d. not detected.

compounds that were characterized as prooxidants and their concentration ranges (in parenthesis) were as follows: dopamine (0.2−2000 μM, max at 10 μM), epicatechin (0.2−500 μM, max at 200 μM), epicatechin-3-gallate (0.1−4 μM, max at 2 μM), epigallocatechin (0.02−100 μM, max at 50 μM), gallic acid (4−10 μM, max at 10 μM), methyl-3,4,5-trihydroxybenzoate (0.2−10 μM, max at 4 μM), n-propyl gallate (0.2−10 μM, max at 4 μM), and quercetin (0.2−2 μM, max at 2 μM). In the same study, the mentioned compounds showed antioxidant activity at higher concentrations.

EGCG is a major catechin in green tea and an effective apoptosis inducing agent.[34] There is growing evidence that catechins can behave as prooxidants and damage cells because of the spontaneous formation of H_2O_2 by polyphenols in solution.[35] Azam et al. (2004)[36] reported that oxidative DNA damage and superoxide anion and hydroxyl radical formation were greater in the case of ECGC than ECAT. In addition, their findings demonstrated that copper-oxidized catechins were more efficient prooxidants as compared with their unoxidized forms,[36] confirming our results. RA has been described as a prooxidant because of its capacity to generate free radicals and H_2O_2[37] in the presence of transition-metal ions.[33,37,38] This compound has two diphenolic rings (A and B), which can be oxidized to their respective o-quinones by autoxidation, generating ROS.[37] Although RES is widely believed to be an antioxidant, there is evidence in the literature to support its prooxidant properties.[39−41] Fukuhara and Miyata (1998)[42] first reported that RES had prooxidant activity in the presence of copper ions, which was further confirmed by the work of Ahmad et al. (2005)[43] showing the formation of RES−Cu(II) complex leading to reduction of cupric ions with concomitant generation of ROS. GSH and other thiol compounds (such as N-acetyl-L-cysteine and mercaptopropionylglycine) are considered to be cellular antioxidants, but GSH may produce thiyl radicals ($GS^{\bullet}$) that potentially cause oxidative damage in biological systems; the formation and reaction of thiyl radicals can occur in many different ways and probably depend on factors such as the nature of the parent thiol compound, local pH, and the presence of O_2 or trace metal ions.[44]

For the statistical comparison of the soluble CEW-AuNC and solid Ca-proteinate Cu(II)−Nc assays, the TPAs of the ECAT standard and the 10-fold diluted artichoke leaf extract were calculated as ECAT equivalents using the ECAT linear regression equation (Table 3). The F-test results given in Table 3 proved that there was no significant difference at 95% confidence level between the precisions of both methods. On the other hand, the arithmetic means in Table 3 may naturally vary, as different antioxidant/prooxidant tests may have different thermodynamics and kinetics.[45]

Table 3. Statistical Comparison (at 95% Confidence Level) of TPA of ECAT Standard and of the 10-Fold Diluted Artichoke Leaf Extract as mg ECAT L^{-1} with Respect to the Cu(II)−Nc Assays Using the CEW-AuNC and Solid Protein-Based Biosensors (N = 5)

sample	parameter	CEW-AuNC-based biosensor	solid protein-based biosensor
ECAT standard	no. of samples	5	5
	average TPA	302.0[a]	210.0[a]
	standard deviation	4.85	3.84
	variance	23.52	14.74
	degrees of freedom	4	
	$F_{calculated}$	1.59	
	$F_{critical}$	6.39	
artichoke leaf extract	no. of samples	5	5
	average TPA	34.42[a]	54.73[a]
	standard deviation	3.18	3.17
	variance	10.11	10.05
	degrees of freedom	4	
	$F_{calculated}$	1.01	
	$F_{critical}$	6.39	

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

2.4. TPA of Synthetic Mixtures. TPAs of prepared synthetic mixtures were found as mM ECAT equivalents by dividing the observed absorbance (A_{450}) to the molar absorptivity of ECAT and compared with those theoretically found (Table 4). The theoretical ECAT equivalent prooxidant

Table 4. Experimental and Theoretical TPA Values of Synthetic Mixture Solutions of Tested Compounds as mM ECAT Equivalent (N = 3)

	Cu(II)−Nc assay using CEW-AuNC	
sample	experimental TPA	theoretical TPA
mixture 1	0.80 ± 0.01	0.73
mixture 2	0.90 ± 0.02	0.96
mixture 3	1.23 ± 0.01	1.30

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activity of a synthetic mixture solution was calculated by summing up the absorbances of concerned compounds and dividing by the molar absorptivity of ECAT. The experimental and theoretical TPA values of the synthetic mixtures were compatible with each other, where these capacities agreed at 95% confidence level ($F_{calculated}$ = 0.197, $F_{critical}$ = 18.513, $F_{calculated} < F_{critical}$ at p = 0.05).

2.5. TPA of Herbal Plant Extracts. Green tea (*Camellia sinensis*), linden (*Tilia*), echinacea (*Echinacea purpurea*), and artichoke leaf (*Cynara scolymus*) were studied as real samples. The major phenolics of mentioned herbal infusions are as follows: in green tea, epicatechin, catechin, epigallocatechin-3-gallate, myricetin, kaempherol, and glycosides of quercetin;[46] in linden, catechin, glycosides of quercetin, glycosides of kaempherol, ester of ferulic acid, and ester of coumaric acid;[46] in echinacea, caffeic acid, neochlorogenic acid, *p*-coumaric acid, ferulic acid, and quercetin;[47] and in artichoke leaf, caffeic acid, chlorogenic acid, narirutin, apigenin 7-rutinoside, cynarin, cynaroside, and glycoside of luteolin.[48] CEW-AuNC and solid protein-based biosensors were applied to these microwave-assisted extracts at different dilution ratios. The TPA values of herbal plant extracts with respect to these assays were expressed as mM ECAT equivalent, shown in Figure 3. The results obtained by both methods were compatible with each other at 95% confidence level (p = 0.05, $F_{calculated}$ = 3.05, $F_{critical}$ = 10.13, $F_{calculated} < F_{critical}$).

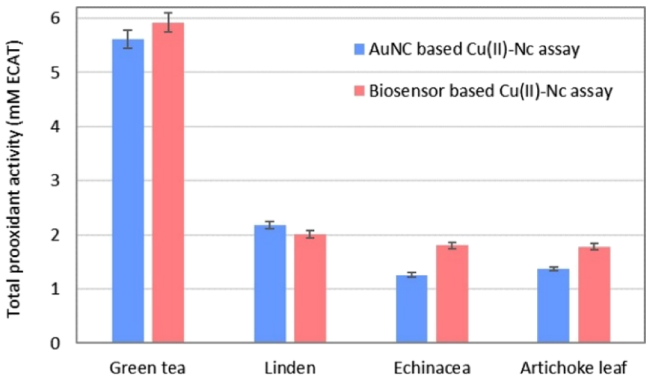

Figure 3. TPAs of herbal plant extracts with respect to the Cu(II)−Nc assays using CEW-AuNC and solid protein-based biosensors (N = 3).

Evaluation of recovery values for the CEW-AuNC-based biosensor was done by spiking two different concentrations of ECAT solution at 0.1 and 0.2 mM to the diluted herbal extracts. Statistical data were given in terms of spiked, theoretical (Theo.), and experimental (Exp.) mM ECAT equivalents in Table 5. The obtained recovery values (Rec.) close to 100% indicated that the CEW-AuNC-based biosensor was reliable to determine the TPA values of plant extracts containing natural antioxidants.

3. CONCLUSIONS

Protein-protected AuNCs were used for the first time in this work in the determination of prooxidant activity of herbal plants. The preparation of our previous calcium proteinate-based biosensor comprised successive steps such as pH adjustment, long-term heating, precipitation, washing, and drying and took about 2 days for complete manufacture. In contrast, the proposed CEW-AuNC biosensor was prepared

Table 5. ECAT Recoveries from Herbal Infusions Using the CEW-AuNC-Based Cu(II)−Nc Assay (N = 3)

herbal plant	added (mM)	Theo. (mM)	Exp. (mM)[a]	Rec. (%)	RSD (%)
1:10 diluted echinacea	0.20, 0.40	0.35, 0.55	0.34 ± 0.01, 0.56 ± 0.01	97, 102	2.9, 1.8
1:10 diluted green tea	0.20, 0.40	0.79, 0.99	0.79 ± 0.03, 1.05 ± 0.01	100, 106	3.8, 0.9
1:5 diluted linden	0.10, 0.20	0.52, 0.62	0.52 ± 0.02, 0.60 ± 0.01	100, 97	3.8, 1.7
1:10 diluted artichoke leaf	0.20, 0.40	0.34, 0.54	0.35 ± 0.02, 0.58 ± 0.01	103, 107	5.7, 1.7

[a](mM ECAT equiv).

within approximately 20 h without any pretreatment. In addition, the procedure took place in solution and was complete in a single incubation step, unlike the other multistep methods (involving procedures of centrifugation, filtration, cleaning, etc.). Therefore, the proposed biosensor contains one-pot biosynthesis and determinations. Although a second 30 min incubation is required for the formation of the Cu(I)−Nc complex in the reference method, the Cu(I)−Nc complex is formed immediately in the proposed method and does not require any incubation process. A lower LOD value for the ECAT compound was obtained with the proposed biosensor than that of the solid protein-based biosensor. As opposed to the solid protein-based biosensor not detecting the prooxidant activities of RA and RES compounds, the corresponding values were perfectly determined by the proposed biosensor. Furthermore, CEW was firmly held by gold NPs (due to protein−thiol bonding to gold) forming a stable sensor while the Ca-proteinate solid had a relatively high ionization constant[49] causing partial leachability of sensor constituents. The developed biosensor was durable, reliable, less expensive, easily applicable, and gave highly reproducible results in reporting the prooxidant activities of antioxidant compounds and herbal extracts. Although it is widely accepted that the antioxidant compounds exhibit health-beneficial effects, they may exhibit prooxidant behavior under certain conditions (with regard to the types and concentrations of antioxidants and transition-metal ions, levels of dissolved O_2 and pH, etc.). It is very important to fully understand the combined antioxidant and prooxidant roles of bioactive substances classified as "antioxidants" so as to monitor the changes observed during the shelf life of food components that may be temporarily subjected to various oxidative stress factors. In conclusion, the developed CEW-AuNC is believed to be the first prooxidant biosensor to allow practical analysis in the solution medium and may be of further use in controlling the composition of special diets as well as the shelf life and oxidative stability of food.

4. EXPERIMENTAL SECTION

4.1. Reagents and Instrumentation. The following chemicals were supplied from the corresponding sources of analytical reagent grade: Nc (2,9-dimethyl-1,10-phenanthroline), RES, and (−)epicatechin (ECAT): Sigma (Taufkirchen, Germany); tetrachloroauric acid solution ($HAuCl_4$) (1.49 M), and RA: Aldrich (Taufkirchen, Germany); EGCG, (+)catechin (CAT) hydrate, AA, GSH reduced, ethanol (EtOH), and sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$): Sigma-Aldrich (Taufkirchen, Germany); EDTA disodium salt dihydrate and copper(II) sulfate: Fluka (Buchs, Switzerland);

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and sodium hydroxide (NaOH), disodium hydrogen phosphate (Na_2HPO_4), and ammonium acetate (NH_4Ac): Riedel-de Haën (Seelze, Germany); calcium chloride: Merck (Darmstadt, Germany). The herbal plants were supplied from a local herbalist.

A Varian Cary 1E UV−vis spectrophotometer (Sydney, Australia) was used for absorbance measurements. Telstar freeze-dryer (Terrassa, Spain) was used to lyophilize the CEW. Inolab pH 7110 pH-meter (Weilheim, Germany) using a glass electrode was used to pH measurements. The incubation solutions were stirred using a Selecta vortex apparatus (Steinhausen, Switzerland). Separation of the precipitated CEW protein was performed using an Electromag M4812PII (Istanbul, Turkey) centrifuge apparatus. A Biosan Multi Bio RS-24 rotator apparatus (Riga, Latvia) was used to stir and incubate. A Bandelin Sonorex ultrasonic bath (Berlin, Germany) was used for the dissolution of weighed chemicals or preparation of herbal extracts. A Memmert water bath apparatus (Schwabach, Germany) was used for incubation process with heating.

4.2. Preparation of Solutions. The NaH_2PO_4/Na_2HPO_4 buffer solution at pH 7.4 (0.2 M), copper(II) sulfate solution (1.0 mM), and NH_4Ac buffer at pH 7.0 (1.0 M) were prepared in distilled water. Nc solution (7.5 mM) was prepared daily in EtOH. The stock solutions of tested phenolic compounds (10 mM) were freshly prepared in EtOH, AA, and GSH in water at the same concentration. The egg white was separated from yolk, lyophilized by a freeze-dryer until completely dry, and then powdered by grinding in a mortar. The protein solution (at 10 mg mL^{-1}) was prepared by dissolving this powder in distilled water.

4.3. Preparation of Synthetic Mixtures. Binary combinations of antioxidant compounds, the concentrations of which were set to remain within the linear concentration ranges of the assay, were prepared for measuring their prooxidant activities as mM ECAT equivalent with respect to the CEW-AuNC-based biosensor. The contents of the binary mixtures were given below:

Mixture 1: 30.7 μM ECAT + 307.7 μM AA.
Mixture 2: 307.7 μM AA + 307.7 μM RA.
Mixture 3: 61.5 μM RES + 615.4 μM GSH.

4.4. Preparation of Herbal Plant Extracts. Herbal plant (green tea, echinacea, linden, and artichoke leaf) extracts were prepared using the microwave-assisted extraction technique. Dried samples (1.0 g) were extracted with 20 mL of aqueous MeOH (80%, v/v) in a microwave oven under stirring. The samples were heated for 3 min to a temperature of 80 °C, kept at 80 °C for 5 min, and finally cooled to room temperature. Before analysis, the herbal plant extracts were filtered through a Gaussian filter/polyester (1.0/0.45 μm) microfilter. The extracts were generally analyzed freshly but were stored at −20 °C in a deep freezer if not instantly analyzed.

4.5. Preparation of a CEW-AuNC-Based Prooxidant Sensor. The CEW-AuNCs were synthesized after a slight modification of the method of Li et al. (2017).[23] Briefly, 25 mL of 10 mg mL^{-1} protein solution was added to 25 mL of 2.5 mM $HAuCl_4$ solution, which was diluted from the stock solution at 1.49 M, and vortexed for 2 min. To the mixture, 3.5 mL of 1 M NaOH solution was added and incubated in a 37 °C water bath for 20 h. After the incubation period, the formed CEW-AuNCs were stored in the refrigerator at 4 °C before use (Figure 4). The formed CEW-AuNC solution was tested for stability and was observed that it preserved its stability after 3 months of storage at +4 °C in a refrigerator.

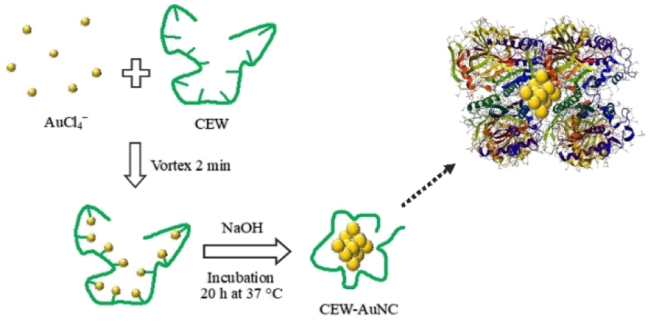

Figure 4. Preparation of the CEW-AuNC-based prooxidant sensor.

4.6. Cu(II)−Nc Assay Using the CEW-AuNC-Based Biosensor. To a test tube were placed 1 mL of CEW-AuNC, 1 mL of NaH_2PO_4/Na_2HPO_4 buffer (pH 7.4), 1 mL of Cu(II) solution (1.0 mM), (x) mL of standard antioxidant or sample solution, and ($2 - x$) mL of distilled water. The test tubes at 5.0 mL were vortexed and shaked at room temperature for 30 min in a rotator. Afterward, 0.5 mL of EDTA solution (0.1 M) was added to eliminate excessive Cu(II) in the medium and vortexed. Both blank and sample solutions contained identical concentration of EDTA. To the mixture, 1 mL of 7.5 mM Nc was added to obtain the Cu(I)−Nc complex and the absorbance at 450 nm was measured against the blank solution prepared under identical conditions excluding the analyte. The linear regression curve (absorbance vs concentration graphs) for each tested antioxidant was constructed under described conditions, and the indirect molar absorptivity of the CEW-AuNC-based Cu(II)−Nc assay for each antioxidants was calculated from the slope of the calibration curve.

4.7. Cu(II)−Nc Assay Using Calcium Proteinate-Based Solid Biosensor. This method,[22] developed by our study group, was based on the measurement of the 450 nm absorbance of the Cu(I)−Nc chelate formed by Nc with a protein-bound copper(I) ion, which was previously shown to mainly bind to the thiol groups of protein. Briefly, the solid prooxidant sensor was incubated with the Cu(II) ion in a medium containing phosphate buffer and antioxidant solution at different concentrations. This was the process required for Cu(II) reduction to Cu(I) and eventual binding to protein thiols. At the end of this period, the aqueous phase was decanted and the solid prooxidant sensor was washed with pure water to eliminate the interference effects that could come from unreacted compounds. During the second incubation step, the Cu(I)−Nc chelate was extracted with ethanolic Nc from a solid protein to the solution phase, and absorbance was measured at 450 nm.

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The authors declare no competing financial interest.

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