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Voltammetry of Adiponectin and its Interactions with Collagen on a Carbon Paste Electrode at Femtogram Level

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Adiponectin, the most abundant protein secreted by white adipose tissue, is known for its involvement in obesity-related disorders such as insulin resistance, type 2 diabetes mellitus and atherosclerosis. Besides its well-known insulin-sensitizing, anti-inflammatory and antiatherosclerotic properties, accumulating evidence suggests that adiponectin may also have anticancer properties and be cardioprotective. The aim of this study is to investigate electrochemical behaviour of adiponectin at carbon paste electrode using adsorptive transfer stripping square wave voltammetry (AdTS SWV). To obtain the most sensitive analytical tool for determination of protein of interest, the time of accumulation on the surface of carbon paste electrode (CPE) and pH of supporting electrolyte (0.2 M phosphate buffer) was optimized. Under the optimized conditions (time of accumulation: 300 s, supporting electrolyte: 0.2 M phosphate buffer pH 6.98), the detection limit (3 signal/noise, S/N) for adiponectin was calculated as 50 pg/ml (250 fg in 5 μl). To investigate the collagen-adiponectin interactions, studying of electroactivity of collagen in CPE was needed. Collagen-modified CPE was prepared simply by mixing a desired quantity of collagen into a conventionally prepared CPE composed of graphite powder and mineral oil. The detection limit (3 signal/noise, S/N) for collagen was calculated as 100 ng/ml. Further, we tested mutual interactions between adiponectin and collagen and found the optimal time of incubation of these proteins as well suggested a possible scheme of their interaction.

Keywords: adiponectin; collagen; interaction; square wave voltammetry; carbon paste electrode

1. INTRODUCTION

Adipose tissue, once viewed as simply a storage and release depot for lipids, is now considered an endocrine tissue [1,2] that secretes various substances (adipokines) including tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), leptin, resistin, visfatin, omenetin, and last but not least adiponectin [3]. Adiponectin (Fig. 1A), the most abundant protein secreted by white adipose tissue, is known for its involvement in obesity-related disorders such as insulin resistance, type 2 *diabetes mellitus* and atherosclerosis [4,5]. Moreover, modulation of the circulating adiponectin concentration is observed in pathologies that are more or less obesity related including the most dangerous cancer [6,7]. Since the initial identification of adiponectin in 1995 [8], almost 6,000 publications have implicated its role in various pathological states. Recent findings suggest that adiponectin may be involved in numerous beneficial biological functions with therapeutic effect. Besides its well-known insulin-sensitizing, anti-inflammatory [9] and antiatherosclerotic properties [10], accumulating evidence suggests that adiponectin may also have anticancer properties and be cardioprotective [11]. A beneficial effect of adiponectin on female reproductive function was also suggested [12]. Due to beneficial effects of this protein, one may suggest to apply it in vascular engineering [13-15].

Enzyme-linked immunosorbent assays (ELISA) is the most common and available method for the widespread measurement of adiponectin in research as well as in clinical practice [16-19]. From the point of view of robust analytical techniques, mass spectrometry was used for determination of this protein [20], however, there is lack other information about analytical chemistry of adiponectin. Considering the assay is time required, quantitative measurement by ELISA is not suitable for large-scale routine laboratory assay. Electrochemistry presents other way to determine adiponectin. Electrochemical methods using hanging mercury drop electrode are convenient for protein detection thanks to their high rapidity, selectivity and ultra-sensitivity (detection limit is down to units of attomoles) [21-50]. However, there is great development in the electrodes based on other materials including carbon due to the possibility to miniaturize whole detection system. Since their invention in 1958, carbon paste electrodes (CPEs) have been utilized for many electroanalytical purposes due to their outstanding properties and have several times reviewed [51-57]. Possibility of their modification by proteins, cell and even tissues is also discussed [58]. This approach has been successfully applied for detection of avidin and/or streptavidin in transgenic plants by using of CPE modified by biotin [59-63]. The aim of this study is to investigate electrochemical behaviour of adiponectin at carbon paste electrode, which has not been done yet. Further, we aim our attention on studying interactions between adiponectin and collagen with respect to use the results obtained in vascular engineering.

2. EXPERIMENTAL PART

2.1 Chemicals and material

The samples of human recombinant adiponectin and collagen were purchased from Vyzkumny ustav pletarsky in Brno, Czech Republic. The primary structure of adiponectin was as follows (the

electroactive tyrosin(Y)/tryptophan(W) is bolded): (1) MLLLGAVLLL LALPGHDQET TTQGPGVLLP LPKGACTG**W**M AGIPGHPGHN GAPGRDGRDG, (61) TPGEKGEKGD PGLIGPKGDI GETGVPGAEG PRGFPGIQGR KGEPGEGA**Y**V **Y**RSAFSVGLE, (121) T**Y**VTIPNMPI RFTKIF**Y**NQQ NH**Y**DGSTGKF HCNIPGL**YY**F A**Y**HITV**Y**MKD VKVSLFKKDK, (181) AMLFT**Y**DQ**Y**Q ENNVDQASGS VLLHLEVGDQ V**W**LQV**Y**GEGE RNGL**Y**ADNDN DSTFTGFLL**Y**, (241) HDTN. Collagen was dissolved in hydrochloric acid (9%) and obtained suspension was transferred into test-tube and vortexed for 15 min at 400 rpm at 4 °C (Vortex 2, USA). Other chemicals used were purchased from Sigma Aldrich (Sigma-Aldrich, USA) unless noted otherwise. Working standard solutions were prepared daily by dilution of the stock solutions with ACS water. The pH value was measured using inoLab Level 3 (Wissenschaftlich-Technische Werkstatten GmbH; Weilheim, Germany). Deionised water underwent demineralization by reverse osmosis using the instruments Aqua Osmotic 02 (Aqua Osmotic, Tisnov, Czech Republic) and then it was subsequently purified using Millipore RG (Millipore Corp., USA, 18 MΩ) – MiliQ water.

2.2. Electrochemical measurement

Electrochemical measurements were performed with AUTOLAB Analyzer (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. Carbon paste electrode was employed as the working electrode. An Ag/AgCl/3M KCl electrode served as the reference electrode. Glassy carbon electrode was used as the auxiliary electrode. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999%) saturated with water for 120 s. Phosphate buffer was used as the supporting electrolyte. Adsorptive transfer stripping square wave voltammetry (AdTS SWV) was performed using the following parameters: initial potential = 0.1 V, end potential = 1.3 V, amplitude = 25 mV, step potential = 5 mV, and frequency = 200 Hz. All experiments were carried out at 25°C. The temperature of supporting electrolyte was maintained by the flow electrochemical cell coupled with thermostat JULABO F12/ED (Labortechnik GmbH, Germany).The raw data were treated using the Savitzky and Golay filter (level 2) and a moving average baseline correction (peak width = 0.05 mV) of the GPES software.

The carbon paste (about 0.5 g) was made of 70% graphite powder (spherical powder, 2-12 μm, 99.95% trace metals basis, Sigma-Aldrich) and 30% mineral oil (*m*/*w*) (free of DNase, RNase, and protease, Sigma-Aldrich). This paste was housed in a Teflon body having a 2.5-mm-diameter disk surface. Prior to measurements, the electrode surface was renewed by polishing with a soft filter paper in preparation for measurement of a sample volume of 5 μl [59,60,62].

3. RESULTS AND DISCUSSION

3.1 Electrochemical behaviour of adiponectin at CPE

Adiponectin is an important protein that contains a diversity of amino acids in its structure (Experimental section, Fig. 1A). From an electrochemical point of view, only tyrosine (**Y**) and

tryptophan (W) have been found to be electroactive using a variety of electrodes [64,65]. Square-wave voltammetric analysis at solid carbon electrodes is not very sensitive and yields only poorly developed signals. However, by using a CPE and sophisticated base line correction, we obtained well-defined voltammetric signals for both Y and W at 0.78 and 0.92 V vs. Ag/AgCl/3 M KCl, respectively, using adsorptive transfer stripping square wave voltammetry (AdTS SWV). A scheme showing the transfer technique is provided in Fig. 1B. The technique is based on the strong adsorption of adiponectin on the electrode surface and subsequent electrode transfer to a washing solution and to an electrolyte not containing any adiponectin in bulk solution where electrochemical measurements were performed. An advantage of using this approach is that only a small amount of sample is required (2-5 µl drop) per voltammetric analysis. Details about these electrochemical transfer techniques were previously described in [66]. Due to the fact adiponectin used in this study contains much more tyrosine residues over tryptophan ones, only Y signal was detected.

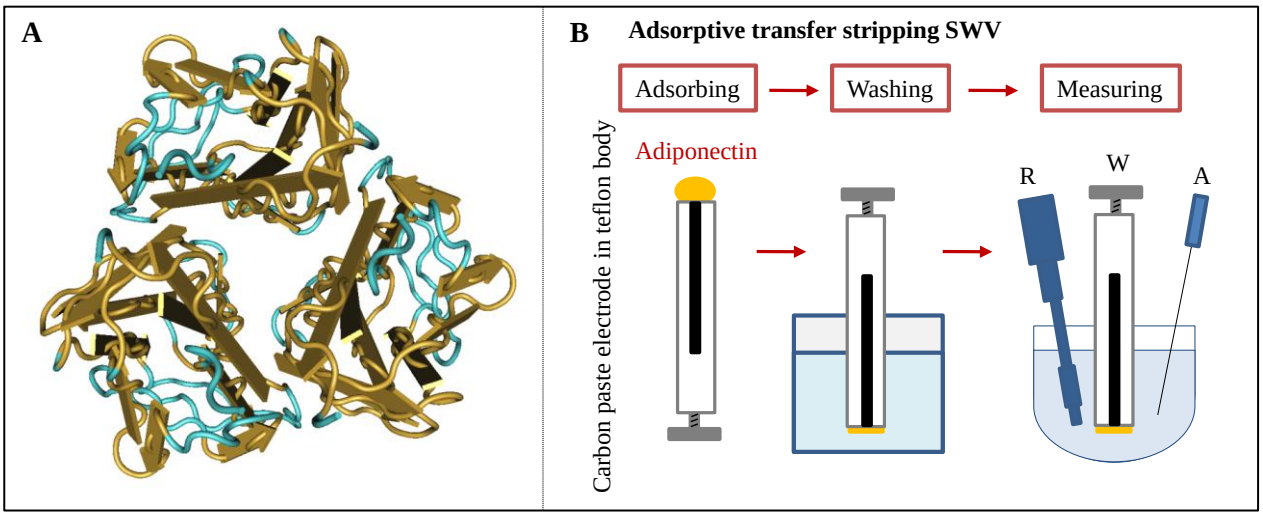

Figure 1. (**A**) Three-dimensional (3D) structure of three units of ACRP30 - adipocyte complement-related protein of 30 kDa or AdipoQ, important adipokine involved in the control of fat metabolism and insulin sensitivity, with direct anti-diabetic, anti-atherogenic and anti-inflammatory activities [67]. (**B**) Scheme of adsorptive transfer stripping (AdTS) technique in connection with the carbon paste electrode (CPE). Primarily, adiponectin is adsorbed on the electrode surface and subsequent the electrode is transferred to a washing solution. Then, the washed electrode is immersed to an electrolyte not containing any adiponectin in bulk solution where an electrochemical measurement is performed.

To obtain the most sensitive analytical tool for determination of protein of interest, the time of accumulation on the surface of CPE and pH of supporting electrolyte (0.2 M phosphate buffer) was optimized. The influence of accumulation time was studied within the interval form 30 to 450 s (Fig. 2A). The Y signal of adiponectin increased with the increasing time of accumulation up to 300 s and then decreased slightly. For better description of covering the surface of an electrode we attempted to divide this dependence into two parts as follows: i) the rising part, time of accumulation from 30 to 300 s and ii) the decreasing part, time of accumulation from 330 to 450 s. If the rising part of this

dependence was plotted linearly, the resulting equation was y = 0.0139x + 0.645. Slope of this dependence (0.0139) reflects the rate of working electrode covering, which can be expressed as 0.1 fg of adiponectin per second. On the hand, the linear regression of the decreasing part of the dependence was y = -0.0027x + 5.409. The decreased can be associated with full coverage of electrode surface and with forming of some higher structure of a protein, which are less electroactive. Moreover, comparing slopes of both dependencies, 0.0139 for increasing part and -0.0027 for decreasing part, the decreasing slope is more five times lower, which shows on good affinity of adiponectin to a surface of CPE. Time of accumulation of 300 s was utilized for optimizing of phosphate buffer pH. The tested pHs were as follows: 6.64; 6.81; 6.98; 7.17; 7.38; 7.73 and 8.04. The Y signal enhanced with the increasing pH up to 6.98 and then decreased (Fig. 2B).

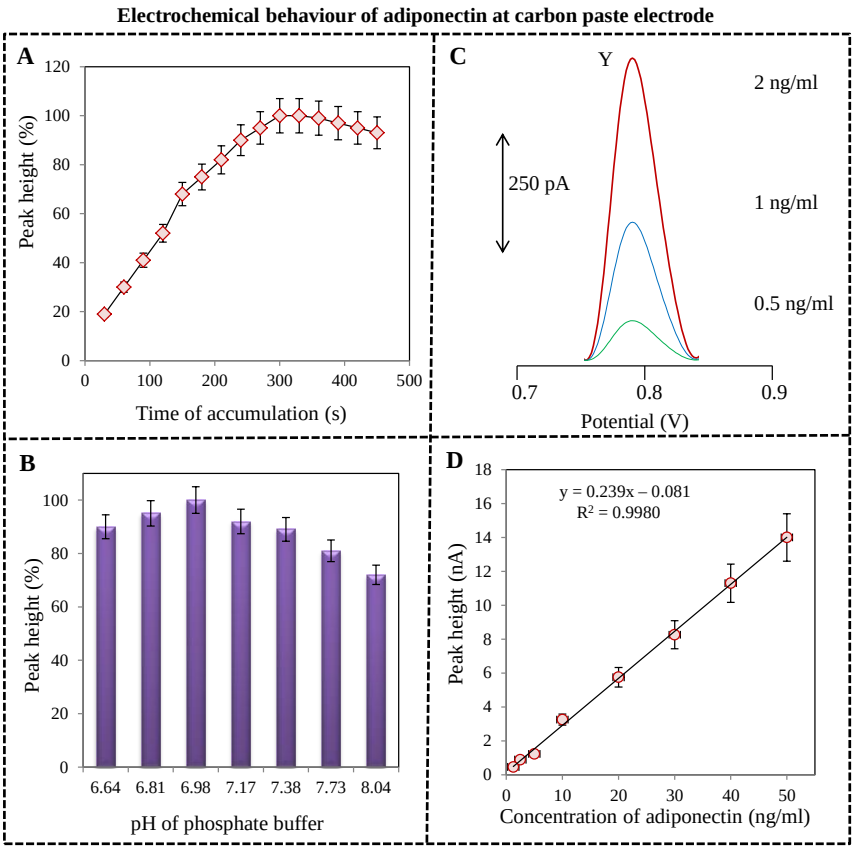

Figure 2. (**A**) The influence of accumulation times (30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420 and 450 s) on the Y signal of adiponectin measured by AdTS SWV. Concentration of adiponectin – 20 ng/ml, supporting electrolyte – 0.2 M phosphate buffer, pH 6.64. Peak of 100 % height corresponds to 4.5 nA. (**B**) The influence of 0.2 M phosphate buffer pHs (6.64, 6.81, 6.98, 7.17, 7.38, 7.73 and 8.04) on the Y signal of adiponectin measured by AdTS SWV. Concentration of adiponectin – 20 ng/ml, accumulation time – 300 s. Peak of 100 % height corresponds to 5.75 nA. (**C**) AdTS SWV signals of adiponectin (0.5, 1 and 2 ng/ml). Accumulation time – 300 s, supporting electrolyte – 0.2 M phosphate buffer, pH 6.98. (**D**) Dependence of Y signal height on concentration of adiponectin. Adsorptive transfer stripping square wave voltammetry (AdTS SWV) was performed using the following parameters: initial potential = 0.1 V, end potential = 1.3 V, amplitude = 25 mV, step potential = 5 mV, and frequency = 200 Hz. All experiments were carried out at 25°C.

The rate of the increasing of signal was 170 pA per 0.1 pH, calculated from the increasing part of the dependence. pH, in which the highest signal was measured, is probably the most convenient for oxidation of tyrosine residues.

There should be also influenced structure of a protein on the surface of CPE, which could also play a role in the electroactivity of the target molecule. Under the optimized conditions (time of accumulation: 300 s, supporting electrolyte: 0.2 M phosphate buffer pH 6.98), the dependence of Y signal height on concentration of adiponectin was measured. AdTS SWV signals of adiponectin (0.5, 1 and 2 ng/ml) are shown in Fig. 2C. The measured dependence was strictly linear over two magnitudes within the interval from 0.5 to 50 ng/ml (Fig. 2D) with relative standard deviation lower than 7 % (n = 3). The detection limit (3 signal/noise, S/N) was calculated according to Long and Winefordner [68], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise. The calculated detection limit for adiponectin was 50 pg/ml, which means 250 fg in 5 µl.

3.2. Collagen analysis

To investigate the collagen-adiponectin interactions, studying of electroactivity of collagen in CPE was needed. Huska et al. and Zitka et al. have investigated the electroactivity of this protein by chronopotentiometric stripping analysis [69] and differential pulse voltammetry Brdicka reaction [70] both at hanging mercury drop electrode, however, to our knowledge, there has not been studied electroactivity of this protein at CPE by using AdTS SWV. Collagen-modified CPE was prepared simply by mixing a desired quantity of collagen into a conventionally prepared CPE composed of graphite powder and mineral oil (Fig. 3A). Compared to CPE of adiponectin, collagen-modified CPE exhibited similar voltammetric signals for Y and W, but we detected Y signal only because of prevalence of this aminoacid in adiponectin. We investigated whether collagen was washed out from the paste by keeping the modified electrode in the electrolyte solution (0.2 M phosphate buffer, pH 6.98). The voltammetric signal decreased app. 10% during an incubation time of 20 min. A measurement is not longer than three minutes, which indicates that this would not markedly affect the peak height of collagen. As expected the voltammetric signal was dependent on the amount of collagen present in the collagen-modified CPE. SWV signals of collagen (5, 10 and 25 µg/ml) are shown in Fig. 3B. A linear increase in peak height was observed with increasing concentration of collagen in the CPE within tested concentration range from 5 to 300 µg/ml (inset in Fig. 3C). The calibration curve for the concentration range examined was linear in the interval from 5 to 100 µg/ml with the regression line of $y = 1.0113x + 33.035$ ($R^2 = 0.9927$) with relative standard deviation lower than 9 % (n = 3) (Fig. 3C). High intercept of the calibration curve can be associated with some unspecific interactions of collagen with carbon paste. Moreover, it can be concluded that 100 µg/ml is enough to fully cover a working electrode surface. The lowest concentration at which the voltammetric signal was detectable under our experimental conditions was 100 ng/ml of collagen in the collagen-modified CPE. The difference between the adiponectin and collagen detection limits might be caused by different arrangement of the analyte on active electrode surface – adsorption vs. incorporation. These results are in good agreement with those of Kizek et al. [60].

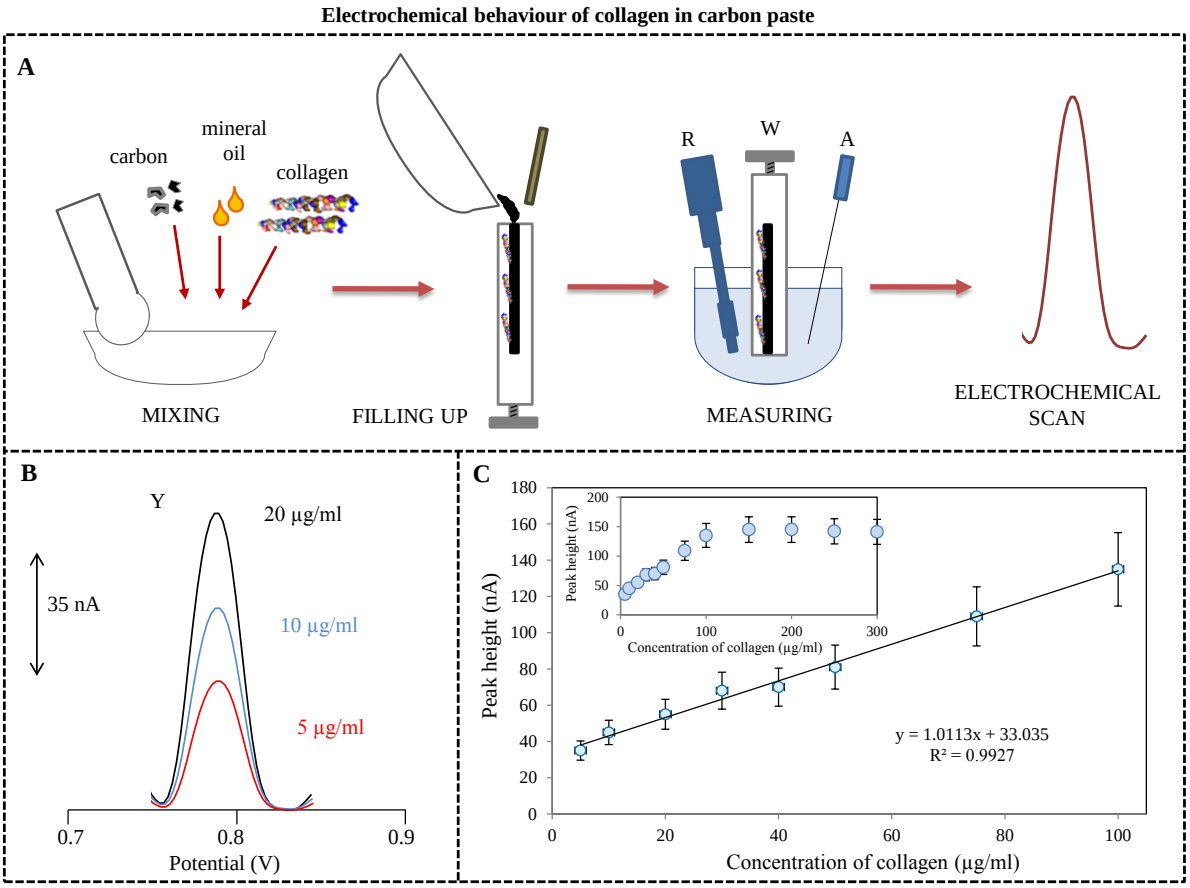

Figure 3. (A) Scheme of collagen-modified CPE; mixing, connection and resulting SW voltammogram of collagen (Y signal only). (B) SWV signals of collagen (5, 10 and 25 µg/ml). (C) Dependence of Y signal height on concentration of collagen within the interval from 5 to 100 µg/ml. For other experimental parameters, see Caption for Figure 2.

3.3. Study of interaction adiponectin:collagen interactions

After the analysis of adiponectin and collagen using carbon paste electrodes, we focused our attention on study of their interaction. Primarily, we tested whether we would be able to follow current changes in the Y signal after the interactions of collagen-modified CPE with adiponectin. Concentration of collagen as 100 µg/ml was used for modification of CPE. We were interested in the issue whether collagen is able to interact with adiponectin forming protein-protein complex or whether adiponectin is adsorbed on the surface of collagen modified electrode without any stronger interaction. Voltammetry is very convenient tool for such types of studies because we are able to determine content of electroactive moieties of forming of more electroactive complexes by measuring of current. The height of carbon paste electrode modified with collagen (100 µg/ml) was 135 nA. To verify type of interaction, adiponectin (30 ng/ml) was applied on the surface of the electrode, incubated (100 min.) and the signal was measured according to scheme shown in Fig. 4A. Briefly, the electrode surface and subsequent electrode transfer to a washing solution and to an electrolyte not containing any adiponectin in bulk solution (0.2 M phosphate buffer, pH 6.98) where electrochemical measurements

were performed. The current of the expected signal, calculated by adding of collagen peak height and adiponectin peak height, is nearly 144 nA. However, the measured signal was 169 ± 9 nA. This is more three time higher enhancement compared to expected value. This shows on the fact that adiponectin interacts with collagen on the surface of CPE and form protein-protein complex, which is more electroactive compared to proteins itself. Based on the obtained results the influence of various concentrations of adiponectin was tested to confirm the interaction between proteins of interest. Tested concentrations of adiponectin were as follows: 1, 5, 10, 20, 30, 40, 50 and 100 ng/ml (Fig. 4B) and the interaction time of 100 minutes was used. The signal increased with the increasing concentration in the tested interval according to the following equation $y = 10.083x + 17.5$, $R^2 = 0.9915$, which confirms our hypothesis on formation of protein-protein complex. To study the stability and structural properties of such complex needs robust analytical instruments.

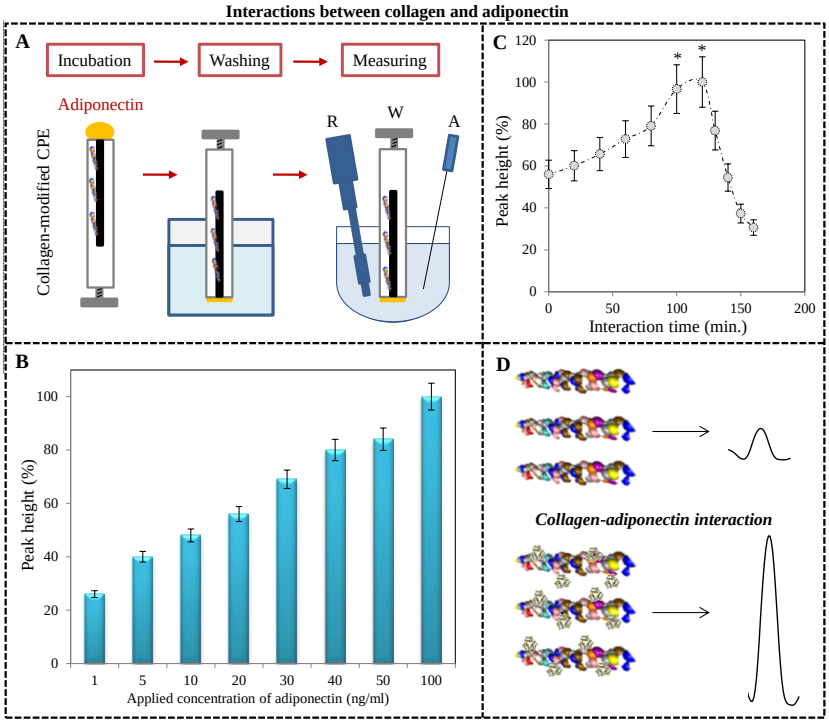

Figure 4. (**A**) Scheme of adsorptive transfer stripping (AdTS) technique in connection with the carbon paste electrode (CPE) for studying of collagen:adiponectin interactions. Primarily, adiponectin is adsorbed on the collagen-modified-electrode surface and subsequent the electrode is transferred to a washing solution. Then, the washed electrode is immersed to an electrolyte not containing any adiponectin in bulk solution where an electrochemical measurement is performed. (**C**) The influence of various concentrations of adiponectin (1, 5, 10, 20, 30, 40, 50 and 100 ng/ml) on Y signal height. Concentration of collagen – 100 µg/ml. Peak of 100 % height corresponds to 50 nA after subtracting of collagen peak of 135 nA. (**B**) The influence of various incubation times (0, 20, 40, 60, 80, 100, 120, 140 and 160) on Y signal height (*$p < 0.05$). Concentration of collagen – 25 µg/ml, concentration of adiponectin – 30 ng/ml. Peak of 100 % height corresponds to 174 nA. (**D**) Possible scheme of interactions between adiponectin and collagen. For other experimental parameters, see Caption for Figure 2.

Further, the influence of various incubation times (0, 20, 40, 60, 80, 100, 120, 140 and 160) was studied (Fig. 4C). Signal of collagen did not change and was subtracted. The measured Y signal steadily increased with time of incubation up to 120 min. and then sharp decrease was observed. The enhancement of the signal is connected with strong interactions of collagen with adiponectin. Data were processed using STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). Statistical significances of the differences interaction times were determined using STATISTICA.CZ. Differences with $p < 0.05$ were considered significant and were determined by using of one way ANOVA test (particularly Scheffe test), which was applied for means comparison. Based on this, we found that Y signal determined after 100 and 120 minutes long incubation time was significantly higher compared to incubation time 0. On the other hand, the decrease can be associated with the collapse of collagen:adiponectin complex and/our with formation of highly complex structures, in which decaying of electroactivity can be expected [71].

4. CONCLUSIONS

Interactions between proteins are important for the majority of biological functions both of pathological and physiological origin. It is not surprising that importance of protein–protein interactions was based for developing of numerous methods to detect them. In this study, we constructed a protein-modified voltammetric bioelectrode by incorporating collagen into a CPE, which allows specific studying of protein-protein interactions. Square-wave voltammetry scans oxidative signals generated from W and Y residues in the proteins, which allows us to determine low concentrations of both proteins (down to pg/ml). Based on the results obtained it can be concluded that collagen strongly interacts with adiponectin, which could be used as a base for fabrication of collagen vascular grafts modified by adiponectin. Considering the molecular weight of both substances possible scheme of their interactions is shown in Fig. 4D. The effect of adiponectin-collagen complexes and properties of this complex could be a base for further studies.

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