Ivan Mikšík[1]
Kateřina Lacinová[1]
Zdeňka Zmatlíková[1,2]
Pavla Sedláková[1]
Vladimír Král[3]
David Sýkora[3]
Pavel Řezanka[3]
Václav Kašička[4]

[1]Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic
[2]Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, Pardubice, Czech Republic
[3]Department of Analytical Chemistry, Institute of Chemical Technology, Prague, Czech Republic
[4]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic , Prague, Czech Republic

Received December 2, 2011
Revised January 6, 2012
Accepted January 6, 2012

Research Article

Open-tubular capillary electrochromatography with bare gold nanoparticles-based stationary phase applied to separation of trypsin digested native and glycated proteins

In this work, open-tubular capillary electrochromatography (OT-CEC) method with bare gold nanoparticles (GNPs)-based stationary phase has been developed and applied for separation of tryptic peptide fragments of native and glycated proteins, bovine serum albumin (BSA), and human transferrin (HTF). The GNPs-based stationary phase was prepared by immobilization of bare GNPs, freshly reduced from tetrachloroaurate(III) ions by citrate reduction, on the sol-gel pretreated inner wall of the fused silica capillary. The separation efficiency, peak capacity, and peptide recovery of this open-tubular capillary column were investigated by varying the experimental parameters such as type and concentration of the buffering constituent and pH of the background electrolyte (BGE), temperature, and separation voltage. The best separations of the above tryptic peptides were achieved in the BGE composed of aqueous 100 mmol/L sodium phosphate buffer, pH 2.5, at separation voltage 10 kV per 47-cm long, 50 μm inside diameter capillary thermostated at 25°C. OT-CEC with bare GNPs stationary phase is shown to be a suitable technique for separation of complex peptide mixtures arising from tryptic digestion of native and glycated BSA and HTF, and for investigation of glycation (nonenzymatic glycosylation) of these proteins.

Keywords: Capillary electrochromatography / Gold nanoparticles / Glycation / Peptide maps / Proteins
DOI 10.1002/jssc.201101049

1 Introduction

In recent years, nanoparticles (NPs), i.e. the particles with spherical-like appearance and with the dimensions in the range of units to hundreds of nanometers, have drawn considerable research attention in many fields including analytical chemistry and separation science [1–6]. Due to their unique physical and chemical properties derived from the "quantum size effect", the NPs are of rising interest in the separation methods. NPs possess a large surface-to-volume ratio, which is important in chromatographic techniques to achieve favorable mass transfer. Within the last decade, the potential of nanostructured materials in separation sciences has gradually been recognized and significant advances have been achieved in gold nanoparticles (GNPs) application in chromatographic and electromigration techniques. NPs have been employed as modifiers of stationary phases in capillary gas chromatography [7, 8] and capillary liquid chromatography (LC) [9–12], as permanent or dynamic internal capillary coatings in open-tubular capillary electrochromatography (OT-CEC) [13–16], as pseudostationary phases in partial filling and continuous filling capillary electrokinetic chromatography [17–19] and capillary electrochromatography (CEC) [11, 17, 20–23]. Among the NPs composed of different inorganic and organic materials (metals, metal oxides, silica, fullerenes, carbon nanotubes, latex, lipids, synthetic polymers, and biopolymers), GNPs belong to the most important ones as evidenced by several reviews dealing with numerous applications of GNPs in separation methods [6, 24–27] as well as with characterization of GNPs by these techniques [28–30].

GNPs applied in separation methods are usually prepared in liquid phase by chemical reduction of gold(III) present in the $H[AuCl_4]$ or $[AuCl_4]^-$ form. This method is simple and controllable to prepare various sizes and shapes of GNPs. The control of size and shape of the particles is achieved via variable experimental conditions (e.g. chemical concentration, surfactants, pH, and temperature) under which the successive growth of Au particles occurs. After dissolving, the solution of tetrachloroaurate(III) salt is rapidly stirred while a suitable reducing agent is added into a reaction mixture, most

Correspondence: Dr. Ivan Mikšík, Institute of Physiology, Academy of Sciences of the Czech Republic, v.v.i., Vídeňská 1083, 142 20 Prague 4, Czech Republic
E-mail: miksik@biomed.cas.cz
Fax: +420-296442558

Abbreviations: **BSA**, bovine serum albumin; **GNP**, gold nanoparticle; **HTF**, human transferrin; **MPTMS**, (3-mercaptopropyl)trimethoxysilane; **NP**, nanoparticle; **OT-CEC**, open-tubular capillary electrochromatography

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often citrate [31], which acts simultaneously as anionic stabilizer of the GNPs. Generally, this method produces modestly monodisperse spherical GNPs suspended in water with a size of about 20 nm in diameter. Larger particles can be produced, but this comes at the cost of monodispersity and shape. GNPs between 16 and 147 nm were obtained via their controlled formation, a method was proposed where the ratio between the reducing/stabilizing agents (the trisodium citrate-to-gold ratio) was varied [32]. Other less frequent reducing agents including carboxylic acids (ascorbic, tannic), alkaline borohydrides, hydrazine, and hydroxylamine are used in the same manner as citrate [24].

CEC is a rapidly emerging analytical technique, which combines the high separation efficiency of capillary electrophoresis (CE) with the selectivity offered by LC. It affords high separation power, high selectivity, short analysis times, good compatibility with mass spectrometry (MS) and low consumption of samples and chemicals [20]. The resulting migration velocity of an analyte through the separation compartment depends not only on its chromatographic retention on the stationary phase, but also on its mobility in the background electrolyte (BGE), on the velocity of electroosmotic flow (EOF), and applied separation voltage.

In OT-CEC, the stationary phase is formed by physically adsorbed or covalently bound compound on the inner wall of the open-tubular fused silica (FS) capillary column [33, 34]. When compared to packed CEC columns, the main advantages of OT-CEC are a higher separation efficiency, simple instrumental handling, and short conditioning times [6]. The first covalent immobilization of GNPs on the prederivatized FS capillaries and their application in OT-CEC was published in 2003 [13]. At first, the GNPs surface was functionalized with dodecanethiol in order to make the surface hydrophobic. Then, these particles were covalently anchored to the prederivatized 3-aminopropyltrimethoxysilane or (3-mercaptopropyl)trimethoxysilane (MPTMS) capillary. This approach was applied to separation of neutral compounds (thiourea, benzophenone, biphenyl, and pyrethroid pesticides), which exhibited repeatable retention times and characteristic reversed-phase behavior. However, there are significant limitations in this application because the phase ratio and sample capacity are relatively low due to the limited amount of stationary phase coating. To overcome these problems, the capillary was etched with ammonium hydrogen difluoride [14]. Then it was prederivatized with MPTMS and finally dodecanethiol-GNPs were immobilized to this modified surface. The etching process was very efficient and increased the surface area by an amplification factor up to 10^3 for the inner capillary wall and, consequently, enhanced interaction between the analytes (thiourea, naphthalene, biphenyl, and polyaromatic hydrocarbons (PAHs)) and the immobilized stationary phase. PAHs can be also separated by the sol-gel technique, in which MPTMS serves as the sol-gel precursor to develop a sol–gel layer on the inner capillary wall prior to assembly of the dodecanethiol-modified GNPs [35]. This approach provides large surface stationary phases with high stability, mass loadability, and separation efficiency and it has been successfully used to analyze drug substances (propiophenone, benzoin, and warfarin). Another method to enhance the phase ratio is self-assembly strategy of GNPs on the inner capillary wall [36, 37]. The covalently immobilized GNPs are either modified with alkanethiols (1-hexanethiol, 1-octanethiol, 1-dodecanethiol, and 1-octadecanethiol) to create a hydrophobic monolayer film or layer-by-layer technology based on the repeated modification of the GNPs surface with 1,9-nonanedithiol, and GNPs are employed to provide a multilayer film on the inner capillary wall [36, 37].

Recently, we have for the first time shown that even nonmodified, bare GNPs immobilized on the sol-gel pretreated FS capillary can serve as a stationary phase for OT-CEC separations of PAHs and peptides [38]. The aim of this work was to extend applicability of this new type of bare GNPs-based stationary phase for OT-CEC separation of complex peptide mixtures, particularly enzymatic (tryptic) hydrolysates of native and glycated proteins, bovine serum albumin (BSA), and human transferrin (HTF), and to use this technique for investigation of glycation (nonenzymatic posttranslational glycosylation) of these proteins.

2 Materials and methods

2.1 Chemicals

Glucose, sodium dihydrogenphosphate, tris(hydroxymethyl) aminomethane-hydrochloride (Tris-HCl), dimethylsulfoxide (DMSO), and toluene were purchased from Lachema (Brno, Czech Republic). Crystallized and lyophilized BSA, HTF, trypsin type IX-S (lot 51K72501), ribose, dithiothreitol, iodoacetic acid, and ammonium bicarbonate were obtained from Sigma (St. Louis, MO, USA). Glyoxal, glutardialdehyde, disodium salt of ethylendiaminetetraacetic acid, and sodium hydroxide were from Merck (Darmstadt, Germany). Potassium tetrachloroaurate(III) and (3-mercaptopropyl)-trimethoxysilane (MPTMS) were from Sigma-Aldrich (Prague, Czech Republic). Guanidine-HCl was obtained from Appli Chem, Biochemica (Darmstadt, Germany) and trisodium citrate dihydrate from Penta (Chrudim, Czech Republic).

2.2 Apparatus and conditions

All CEC and capillary zone electrophoresis (CZE) experiments were performed on Beckman P/ACE analyzers, OT-CEC separations with GNPs-coated FS capillary on the system 5500, and CZE analyses with bare FS capillary on the system 5000 (Beckman Coulter, Fullerton, CA, USA). In both cases, FS capillaries of 47 cm total length, 40 cm effective length (to the detector), inside diameter 50 μm, outside diameter 375 μm, were used. Detection was done by UV-absorbance recording at 214 nm. The sample was injected hydrodynamically (3.45 kPa, 10 s). The analyses were run at 25°C, at separation voltage of 10 kV. At the start of the analysis, the

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capillary was washed with the BGE for 3 min. The BGEs were prepared from the deionized water and particular chemicals and filtered through the 0.45 μm filter (Millipore, Bedford, MA, USA) prior to use. DMSO was used as an EOF marker.

2.3 Preparation of the GNPs

Our approach for GNPs preparation was based on the Au(III) reduction by citrate according to Turkevich et al. [31]. The detailed description of GNPs preparation can be found in our previous paper [39]. Shortly, 1 mL of 1% (m/v) aqueous solution of the potassium tetrachloroaurate(III) and 2.5 mL of 1% (m/v) aqueous solution of the trisodium citrate dihydrate were added to 100 mL of boiling water (under reflux). Heating was continued for 10 min; during this time, the solution color changed from pale yellow to gray-blue, then to purple, and finally to wine-red. Reaction vessel was then allowed to cool to room temperature.

2.4 Preparation of the sol-gel capillary with GNPs stationary phase

A sol-gel pretreated FS capillary was prepared according to a procedure described in detail elsewhere [40]. Briefly, 50-cm long capillary was rinsed (16.6 μL/min) with 1 mL NaOH (1 mol/L) followed with 1 mL of deionized water (16.6 μL/min) and drying at 180°C overnight. The capillary was then rinsed (20 μL/min) with 100 μL of MPTMS/EtOH(96%)/HCl (0.01 mol/L) solution (7:2:1, v/v/v), which was previously stirred for 24 h. The capillary filled with this solution was allowed to stay for 2 h at room temperature. The excess of the solution was forced out of the capillary under argon pressure (500 kPa) for 10 min followed by drying at 120°C overnight. After that, the capillary was rinsed (20 μL/min) with 0.5 mL acetone and 0.5 mL methanol followed by drying at room temperature by argon flow (500 kPa) for 15 min. GNPs-based stationary phase was prepared by washing of the pretreated capillary with 2 mL of freshly prepared GNPs solution at flow rate 16.6 μL/min and keeping the capillary to stand for 2 days with both ends submerged in this solution. Finally, 1 mL of ultrapure water was pumped (20 μL/min) through the capillary and then the GNPs modified FS capillary was ready for OT-CEC analyses.

2.5 Characterization of GNPs

For characterization of GNPs, UV-Vis spectra were measured using a Cary 400 SCAN UV-Vis spectrophotometer (Varian, Palo Alto, CA, USA) and transmission electron microscopy images were obtained from a JEM-3010 microscope (Jeol, Tokyo, Japan). Detailed procedure is described in previous papers [38, 40].

The inductively coupled plasma mass spectrometry (ICP-MS) measurements of the amount of gold immobilized in the sol-gel pretreated FS capillary were carried out using an Elan DRC-e spectrometer (Perkin Elmer, Ontario, Canada) equipped with Meinhard nebulizer (Meinhard, Golden, CO, USA), a cyclonic spray chamber, and Gilson 212 peristaltic pump (Gilson, Inc., Middleton, WI, USA). Quantification of gold was based on ^{197}Au. Stock solution (SS) (1 mg/mL) of Au was prepared by dilution of a standard solution 1.000 ± 0.002 g/L Au (Merck). SS (2 mg/mL) of Bi (used as the internal standard (IS)) was prepared by dilution of a standard solution 1.000 ± 0.002 g/L Bi (Merck). Both solutions were acidified with nitric acid (5 mL/100 mL) and final volume was adjusted with ultrapure water. Calibration solutions in water were prepared into 50 mL volumetric flasks using 0, 0.1, 0.25, 0.5, and 1 mL of the SS solution; 1 mL of IS solution; and 2 mL of HNO_3. The resulting concentrations of the calibration solutions were 0, 2, 5, 10, and 20 ng/mL.

The amount of gold loaded in the capillaries was measured in solutions prepared by the following procedure. First, the polyimide capillary coating was removed by hot concentrated sulfuric acid. Then the capillary was completely dissolved in 3 mL of concentrated hydrofluoric acid and the resulting solution was evaporated to dryness. Next, 4 mL of concentrated nitric acid and hydrochloric acid mixture (1:3, v/v) was added to the residue of the capillary sample to dissolve gold and the solution was again evaporated to dryness. Finally, the residue was dissolved in 1 mL of nitric acid and transferred into the 25 mL volumetric flasks, to which 0.5 mL of IS solution was added and volume adjusted to 25 mL with water.

2.6 Preparation of native and glycated proteins and their tryptic digests

Glycated proteins, BSA and HTF, were prepared as follows: proteins were dissolved in phosphate buffer (0.2 mol/L NaH_2PO_4; pH 7.4) to a final concentration of 1 mg/mL. Four solutions of each protein (10 mL) were incubated (at 37°C, 7 days) with one of the following selected substances containing oxo-group (glucose, ribose, glyoxal, and glutardialdehyde), whose concentration was 0.1 mol/L in the solution of protein. Control samples of native proteins were prepared in the same way as glycated proteins with the exception that no oxo-compound was added. To prevent contamination during protein incubation with oxo-compounds, sodium azide and a thin layer of toluene were added to each sample. Dialysis followed after incubation and lasted 24 h. The dialyzed samples were lyophilized.

Half a milliliter of pH 8.4 buffer (6 mol/L guanidine HCl; 1.2 mol/L Tris-HCl; 2.5 mmol/L Na_2EDTA) was added to 5 mg of lyophilized samples of native and glycated BSA and HTF. Reduction was performed by adding 25 μL of 1 mol/L dithiothreitol. Samples were incubated for 30 min at 65°C. The subsequent alkylation took place using 60 μL of 1 mol/L iodoacetic acid (incubation at room temperature, 40 min in the dark). This reaction was stopped by adding 15 μL of 1 mol/L dithiothreitol.

Reduced and alkylated native and glycated proteins were desalted in Econo-Pac 10 DG columns (Bio-Rad Laboratories, Hercules, CA, USA) and lyophilized. Desalted BSA and

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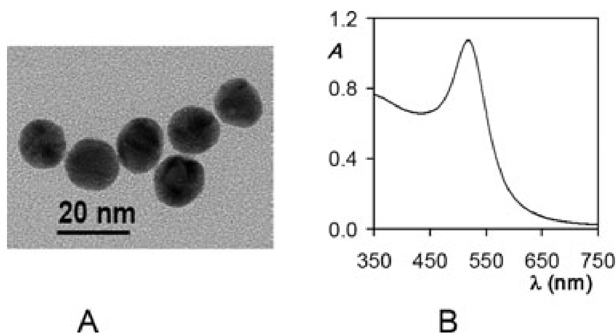

Figure 1. Transmission electron microscopy image (A) and UV-Vis spectrum (B) of the freshly prepared GNPs.

HTF samples were digested by trypsin. First, the protein samples were dissolved to a concentration of 5 mg/mL with 20 mmol/L ammonium bicarbonate buffer (pH 7.8) and then treated with trypsin (1:50 (mass/mass) enzyme/substrate ratio). Incubation was done at 37°C for 2 h. Blank samples were prepared by incubating the enzyme solution alone under identical conditions.

3 Results and discussion

3.1 Preparation and characterization of the GNPs

The preparation procedure for the GNPs resulted in spherical NPs with average diameter of 14.7 nm (Fig. 1A) at concentration 2.75 nmol/L, which exhibited plasmon resonance band at 518 nm (Fig. 1B). ICP-MS was used for the quantification of gold bonded onto the capillary inner wall. The amount of gold found corresponded to 160 ng/m of the capillary [38].

3.2 Development of OT-CEC and CZE methods for peptide mapping of proteins

Tryptic peptides of native BSA and HTF have been used as model samples for the development of suitable experimental conditions for OT-CEC and CZE methods for peptide mapping of both native and glycated BSA and HTF proteins. Several types of the BGEs with different buffering constituents (phosphates or borates), variable concentration (5, 10, 20, 50, and 100 mmol/L), and pH (2.5, 7.0, and 9.2) were tested. Representative examples of OT-CEC separations of BSA tryptic peptides in phosphate buffers at pH 2.5 and 7.0 and at concentrations 20 mmol/L and 100 mmol/L, and in the borate buffer at pH 9.2 and concentration 20 mmol/L are presented in Fig. 2. As can be seen from these records, separations in neutral and alkaline BGEs were worse than those at acidic BGEs and separation at high ionic strength of 100 mmol/L was better than those at low ionic strength of 20 mmol/L. Better separation at high ionic strength can be attributed to the lower electromigration dispersion. In general, the electromigration dispersion is lower at higher concentration ratio of the BGE versus analyte. The lower separation efficiency and resolving

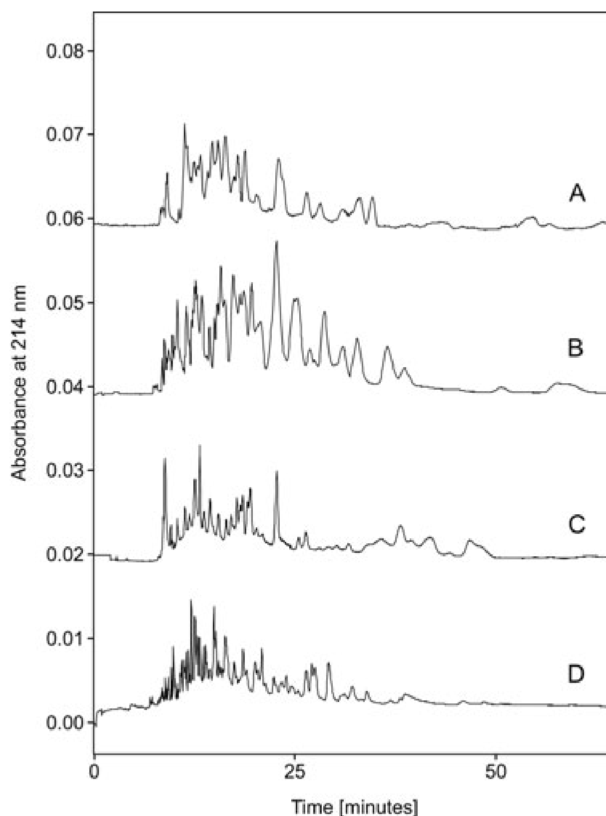

Figure 2. Separation of tryptic peptides of native BSA by OT-CEC in GNPs coated FS capillary using different BGEs. (A) 20 mmol/L sodium phosphate, pH 7.0; (B) 20 mmol/L sodium phosphate, pH 2.5; (C) 20 mmol/L sodium borate, pH 9.2; (D) 100 mmol/L sodium phosphate, pH 2.5. For other experimental conditions, see section 2.2.

power of neutral and alkaline BGEs is probably caused by sticking of peptides to the capillary wall. Nevertheless, the sticking of peptides at these neutral and alkaline pH values on the GNPs-coated FS capillary was lower as compared to sticking of peptides on the inner wall of the bare FS capillaries in the CZE mode (data not shown). From the above and other BGEs used, the best separation efficiency and the highest number of resolved peptides (peak capacity) was achieved in the BGE composed of 100 mmol/L sodium phosphate buffer, pH 2.5. Hence, this BGE has been used for all subsequent experiments with tryptic digests of native and glycated BSA. This BGE was found to be the best also for separation of tryptic peptides of both native and glycated HTF.

3.3 Comparison of CZE and OT-CEC separations of tryptic peptides of native proteins

A comparison of separation of tryptic digest of native BSA in the uncoated FS capillary by CZE (record A) and in the GNPs-coated capillary by OT-CEC (record B) in the optimized 100 mmol/L sodium phosphate BGE, pH 2.5, is shown in Fig. 3A. Apparently, the capillary coated with GNPs provided different separation as compared to bare FS capillary. The

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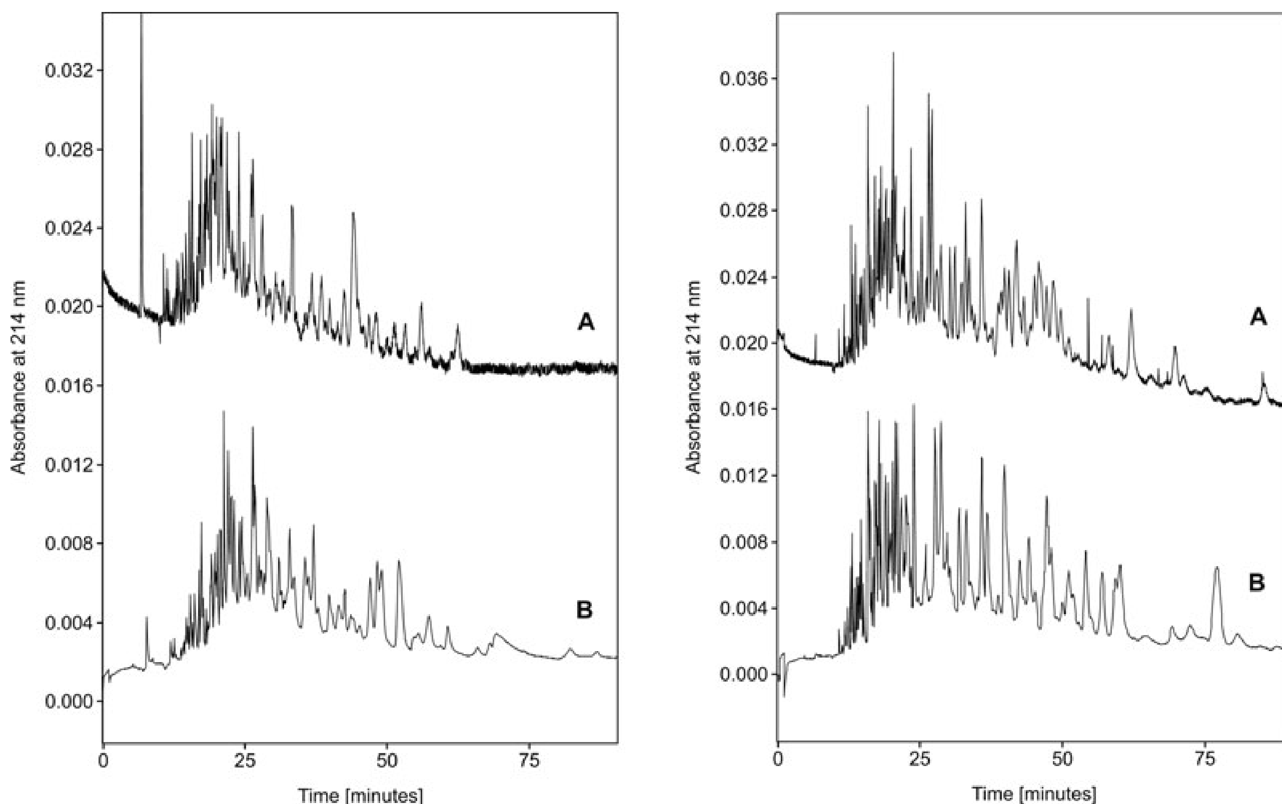

Figure 3. **(A = left figure)** Separation of tryptic peptides of native BSA by CZE in bare FS capillary (A) and by OT-CEC in GNPs coated FS capillary (B). In both cases, the BGE was 100 mmol/L sodium phosphate, pH 2.5. Injection time 10 s, 3.45 kPa injection pressure. For other experimental conditions, see Section 2.2. **(B = right figure)** Separation of tryptic peptides of native HTF by CZE in bare FS capillary (A) and by OT-CEC in GNPs coated FS capillary (B). Other conditions as in Fig. 3A.

differences in these two separation patterns show that at least some of the BSA tryptic peptides interact with GNPs stationary phase and confirm another, mixed chromatographic and electrophoretic (OT-CEC) separation mechanism involved in GNPs-coated capillary. Similar significant differences have been observed in the CZE and OT-CEC peak profiling of tryptic digest of HTF, see Fig. 3B. In both cases, the differences in migration times were influenced also by different EOF, which was almost two times faster in the GNPs-coated capillary with 106-min migration time of EOF marker, DMSO, than in the bare FS capillary with 198-min migration time of DMSO.

The increased separation power of OT-CEC with GNPs-based stationary phase as compared to CZE in bare FS capillary can be demonstrated by the higher number of resolved peaks obtained by the former technique: in the case of tryptic peptides of BSA, 65 peaks by OT-CEC versus 51 peaks by CZE; in the case of HTF, 67 peaks by OT-CEC versus 58 peaks by CZE.

3.4 OT-CEC separations of tryptic peptides of native and glycated proteins

The newly developed OT-CEC method with bare GNPs-based stationary phase has been employed to the investigation of posttranslational modifications of proteins, particularly for analysis of glycation products BSA and HTF resulting from the reactions of these proteins with low molecular mass oxo-compounds, glucose, ribose, glyoxal, and glutardialdehyde. OT-CEC separations of tryptic digest of BSA glycated by glucose, ribose, glyoxal, or glutardialdehyde in 100 mmol/L sodium phosphate BGE, pH 2.5, provided well-resolved complex peptide maps with significant differences for the BSA glycation by the particular oxo-compounds, see Fig. 4. As expected, the extent of the modifications depended on the nature of the modifiers used. Glucose, being a relatively mild modifier, offered a rich profile of tryptic peptides of BSA similar to that of native BSA (see Figs. 4A and B) while reaction with ribose led to a moderately modified protein, more resistant to enzymatic cleavage and providing a lower number of well-resolved peaks (see Fig. 4C). The treatment of BSA with the most reactive dialdehydes, glyoxal, and glutaraldehyde, resulted in cross-linked proteins, highly resistant to trypsin cleavage and providing, especially in the case of glutaraldehyde, rather limited number of sufficiently high and well-resolved peaks, see Figs. 4D and E. This behavior can be explained by the well-known rather strong (up to irreversible) interactions of larger polypeptide fragments and proteins with the GNPs stationary phase and/or noncoated parts of the FS capillary wall. Also, protein modifications may alter the properties of the proteins in such a way that the

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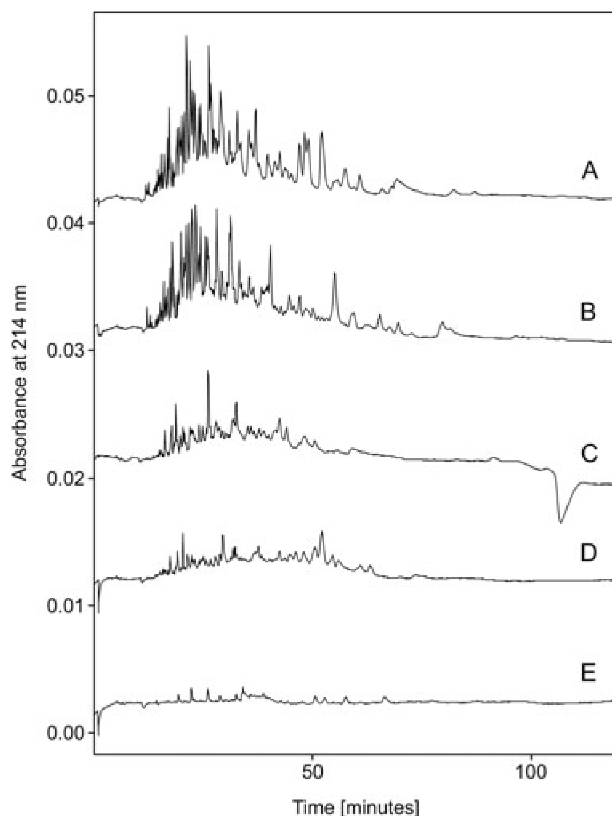

Figure 4. OT-CEC tryptic peptide maps of native BSA (A), and of BSA modified by glucose (B), ribose (C), glyoxal (D), and glutardialdehyde (E). Other conditions as in Fig. 3A.

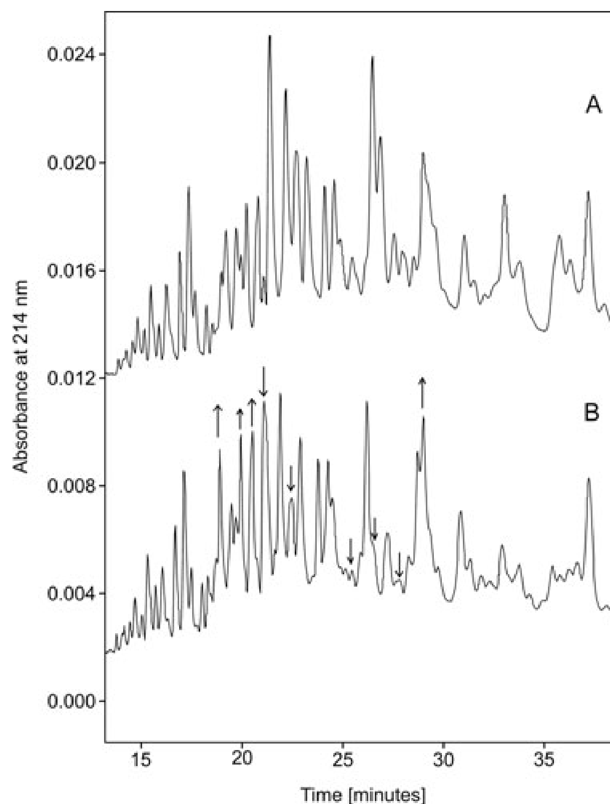

Figure 5. Enlarged sections of the OT-CEC tryptic peptide maps of native BSA (A), and BSA modified by glucose (B). Other conditions as in Fig. 3A. The arrows above the peaks of peptides of BSA treated by glucose indicate 50% decrease or increase in peak area of these peaks as compared to the peaks of peptides of native BSA.

interactions of the generated tryptic peptides to the GNPs stationary phase or noncoated capillary wall are stronger. In the case of the relatively mild effect of glucose modification, this effect is probably less profound. It appears feasible to propose that the monotopical modifications possess a smaller deteriorative effect on the arising profile than inter- and intramolecular cross-linking of proteins by bifunctional agents.

Modification of BSA by glucose and ribose rearranged the OT-CEC profiles of their tryptic peptide maps in such a way that the main changes were mostly present in the front-end part of the OT-CEC runs. In the retention time window of ca 10–40 min, nine relevant changes (differences in peak areas at least 50%) were observed in the peak profiles of OT-CEC separations of tryptic peptides of native of glucose modified BSA (see Fig. 5) and 18 such changes were found in the profiles of tryptic peptides of native and ribose treated BSA (see Fig. 6).

OT-CEC with GNPs stationary phase has been applied also to separations of tryptic peptide fragments of native HTF and HTF glycated by glucose, ribose, glyoxal, and glutardialdehyde, see Fig. 7. From the presented electrochromatograms, we can see that the peptide maps of native HTF and HTF glycated by oxo-compounds are qualitatively similar to those of native and glycated BSA. The changes induced by HTF modification with glucose and ribose (shown in Figs. 7B and C) were much less expressed than those induced by bifunctional agents, glyoxal, and glutaraldehyde, presented in the Figs. 7D and E, respectively. Comparison of enlarged front-end parts of the records of OT-CEC separations of tryptic digest of native HTF and HTF modified by glucose presented in the Fig. 8 revealed 20 differences in the peak profiles (changes at least 50% of peak area) of these two samples. Similarly, 15 significant changes were found in the profiles of HTF glycated by ribose versus glucose (Fig. 9). In the peptide map of ribose-modified HTF (Fig. 9B), a new peak with migration/retention time at 22.6 min has appeared and an absence of peak with migration/retention time 25.7 min was observed as compared to peptide map of glucose modified HTF. The repeatability of migration times was about 2–5% for BSA, and the stability of GNPs-coated capillary was enough for at least 130 analyses.

It is worth to mention that the separation of native and modified peptides and proteins is a very important issue for the investigation of posttranslational modifications of proteins and it is also a challenging task for analytical methods since it is necessary to analyze complex mixtures of modified and native peptides/proteins even if the modifications are

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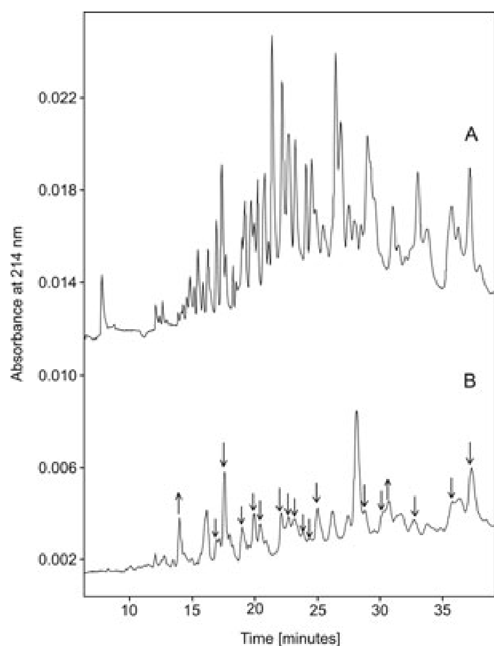

Figure 6. Enlarged sections of the OT-CEC tryptic peptide maps of native BSA (A), and BSA modified by ribose (B). Other conditions as in Fig. 3A. The arrows above the peaks of peptides of BSA treated by ribose indicate 50% decrease or increase in peak area of these peaks as compared to the peaks of peptides of native BSA.

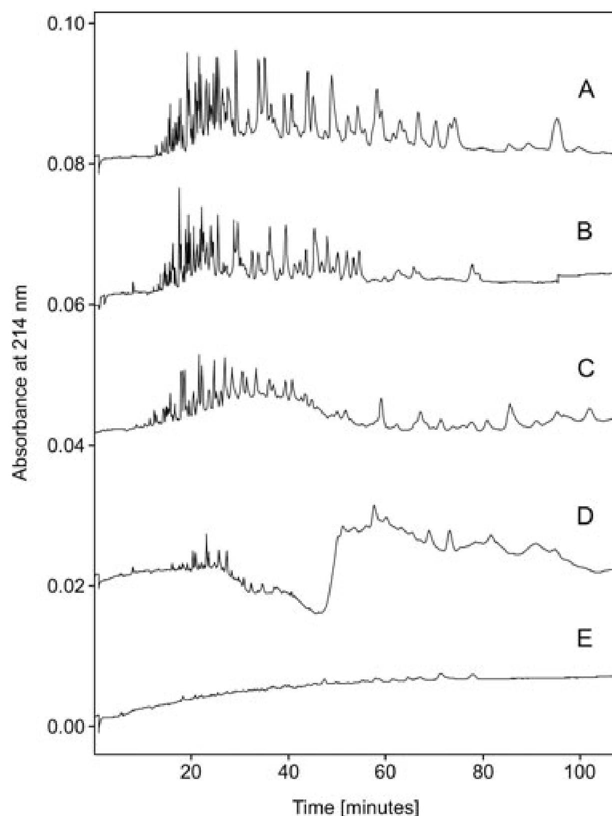

Figure 7. OT-CEC tryptic peptide maps of native HTF (A), and HTF modified by glucose (B), ribose (C), glyoxal (D), and glutardialdehyde (E). Other conditions as in Fig. 3A.

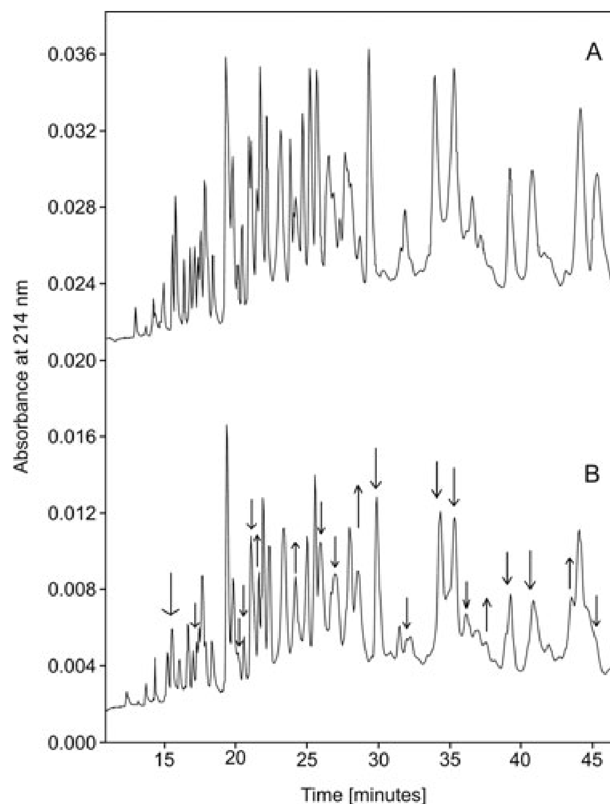

Figure 8. Enlarged sections of the OT-CEC tryptic peptide maps of native HTF (A), and HTF modified by glucose (B). Other conditions as in Fig. 3A. The arrows above the peaks of peptides of HTF treated by glucose indicate 50% decrease or increase in peak area of these peaks as compared to the peaks of peptides of native HTF.

present at low frequency and only a small amount of material is available. For this reason, a plethora of high-resolution separation techniques is necessary that allow to separate these complex mixtures and to distinguish the peptide profiles of native and modified proteins. We experimentally proved that OT-CEC with GNPs-based stationary phase is able to separate complex mixtures of tryptic peptides of BSA and HTF and to provide alternative pattern of peptide maps of these proteins as compared to those obtained by other high-performance separation and detection techniques, such as HPLC-MS and CZE-MS [41] and OT-CEC with salophene–lanthanide–Zn^{2+} complex-based stationary phase [42]. Thus, OT-CEC with GNPs stationary phase has a potential to broaden the family of techniques suitable for separation of peptides and proteins and for investigation of the posttranslational modifications of proteins. However, further developments will be necessary for coupling of this technique with MS detection, which could be able to elucidate the structure of the modified peptides. Partial success in this long-term research has been already achieved when carboxymethylation of lysine was determined as the main modification of BSA treated with glucose and ribose using HPLC-MS and CE-MS analyses of tryptic digests of native and glycated BSA [41].

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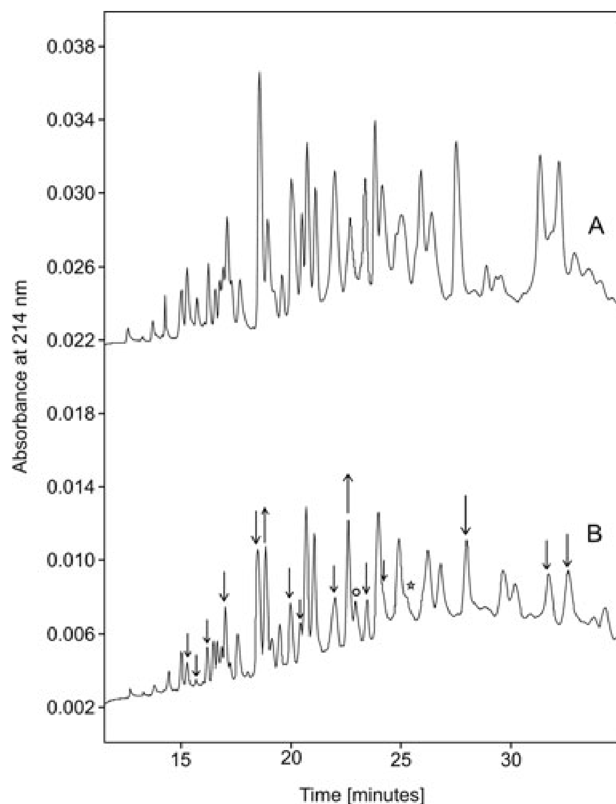

Figure 9. Enlarged sections of the OT-CEC tryptic peptide maps of HTF modified by glucose (A), and HTF modified by ribose (B). Other conditions as in Fig. 3A. The arrows above the peaks of peptides of HTF treated by ribose indicate 50% decrease or increase in peak area of these peaks as compared to the peaks of peptides of HTF modified by glucose. The circle indicates a new peak arising at 22.6 min and the asterisk indicates missing peak at 27.5 min.

4 Concluding remarks

In this work, a new technique of OT-CEC with bare GNPs-based stationary phase was shown to be able to separate complex mixtures of tryptic peptides of native and glycated BSA and HTF. The mildest modification of these proteins was observed after their treatment with glucose; in this case, presumably only monofunctional derivatives were formed. On the other hand, the most intense modifications were found after BSA and HTF reaction with glutardialdehyde, which resulted in high degree of both inter- and intramolecular cross-linking of these proteins. Thus, OT-CEC with bare GNPs-based stationary phase proved to possess potential to become a suitable tool for investigation of nonenzymatic as well as enzymatic posttranslational modification of proteins.

This work was supported by the Czech Science Foundation, grants nos. 203/09/0675 and 203/08/1428, and by the Research Projects AV0Z50110509 and AV0Z40550506 of the Academy of Sciences of the Czech Republic.

The authors have declared no conflict of interest.

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