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Research Article

Gold nanoparticle-coated capillaries for protein and peptide analysis on open-tubular capillary electrochromatography

We report a new method of immobilization of gold nanoparticles (AuNPs) on a fused-silica capillary through covalent binding. The resulting modified capillary was applied to electrophoretic systems to improve the efficiency of separation and the selectivity of selected solutes. The immobilization of AuNPs on the capillary wall was performed in a very simple and fast way without requiring heating. The surface features of an AuNP-coated capillary column were determined using the scanning electron microscopy. The chromatographic properties of AuNP-coated capillaries were investigated through variation of the buffer pH and separation voltage. Effective separations of synthetic peptides mixture were obtained on the AuNP-coated capillaries. The method shows a remarkable stability since it was reused about 900 times. The capacity factor was duplicated. Therefore, this modification is stable and can be applied to different separation purposes. A complex mixture of tryptic peptide fragments of HSA was analyzed in both the bare- and the AuNP-coated capillaries. Better electrophoretic peptide profile was observed when using the AuNP-coated capillary.

Keywords:
Gold nanoparticles / Open-tubular capillary electrochromatography / Peptides / Separation techniques

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1 Introduction

In the last years, the development of proteomics induced a growing interest in the structural analysis of proteins. After the course to decrypt nucleic acids coding sequences, the research community focuses on uncovering protein sequences and consequently, designs methods to achieve this goal [1]. Electrophoretic techniques have proven to be successful to study peptides and proteins; the separation profile responses to the charge-to-mass ratio, when applying a potential difference. However, there are some limitations with CE, such as the inability to separate nonionic solutes and analytes with the same size-to-charge ratio. Some of them can be solved with packed capillaries, resulting in versatile techniques that combine the efficiency of CE with the selectivity of liquid chromatography [2–4].

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Abbreviations: A, angiotensin I; **APTES,** amino propyltriethoxy silane; **AuNPs,** gold nanoparticles; **B,** bradykinin; **L,** luteinizing hormone-releasing hormone; **M,** methionine-enkephalin; **N_2,** nitrogen gas; **O,** oxytocin; **OT-CEC,** open-tubular capillary electrochromatography

The nanosized particles have unique physical and chemical properties relative to the bulk material due to a large surface-to-volume ratio derived in the "quantum size effect" [5]. They have been the focus of extensive attention in various fields of chemistry, physics, materials, and medicine. In separation science, polymeric nanoparticles acting as a pseudostationary phase were applied to CEC in filling experiments [6–11]. Peptides and proteins were separated by CE in fused-silica capillaries coated with an irreversibly adsorbed monolayer of derivatized polystyrene nanoparticles [11, 12].

The interaction of peptides and proteins with gold surfaces has been studied in the context of biomimetic chemistry [13]. These biomolecules containing thiol (SH) or amino (NH_2) groups can spontaneously be adsorbed onto the metallic surface to generate well-organized, self-assembled monolayers [14–19], through noncovalent binding forces, such as electrostatic and hydrophobic interactions, hydrogen bonding, and π–π stacking. For this reason, the mobility of peptides and proteins result significantly altered by the presence of gold nano-particles (AuNPs) with the improvement of efficiency in separation, stacking, and analysis time [20]. The efficient separation of acidic and basic proteins was achieved using didodecyldimethylammonium bromide (DDAB), bilayer-protected AuNPs as dynamic coating additives [21]. Recently, a modified capillary wall by the ionic adsorption of AuNPs on a polyelectrolyte multilayer (PEM) was reported as a new medium for

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the separation of neutral analytes and proteins in open-tube capillary electrochromatography (OT-CEC) [22].

The use of additives in the buffer, such as surfactants, cyclodextrins, or nanoparticles, can increase the background noise introducing problems in the trace analysis [23]. For this reason, we decide to explore the immobilization of AuNPs on the capillary inner surface in order to design a new OT-CEC method for peptides and proteins analysis.

We have previously informed the modification of capillary wall by coating the fused silica with metalloporphyrins [24, 25], resulting in an efficient method for peptide separation.

Herein, we report a simple method for the AuNPs coating of a fused-silica capillary and evaluate the impact of this coating on EOF and separation. The resolution, sensitivity, and reproducibility of the OT-CEC method were optimized.

2 Materials and methods

2.1 Chemicals

Analytical-grade gold(III) chloride trihydrate salt (Aldrich) and sodium citrate were used as supplied. 3-Amino propyltriethoxy silane (APTES) was purchased from Fluka. All other reagents came from Merck and used as received. Water was deionized and filtered using a Millipore water purification system (18 MΩ), from Barnstead-Thermolyne (Dubuque, IA, USA).

The bioactive synthetic peptides, bradykinin (B), angiotensin I (A), luteinizing hormone-releasing hormone (L), oxytocin (O), and methionine-enkephalin (M), were obtained from Sigma-Aldrich (St. Louis, MO, USA). Amino acid sequence, relative molecular mass, and isoelectric point of each peptide are listed in Table 1. HSA was purchased from the Institute of Hematology and Hemotherapy (University of Córdoba, Córdoba, Argentina).

2.2 Instrumentation

Separations were performed with a P/ACE MDQ (Beckman Coulter, Brea, CA, USA), equipped with a UV detector. Data were processed by the 32 Karat™ software (Beckman Coulter). For all experiments, the CE system temperature was held at 25°C, and UV-detection at 214 nm (deuterium lamp) was performed. The fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) had a 75-μm id and a 375-μm od. The total length of the capillary was 41.2 cm, with a distance of 31 cm between the injection and the detection window. All solvents and solutions for the CEC analysis were filtered through a 0.45-μm nylon membrane filters to remove particulate matter, from Micro Separation (Westboro, MA, USA).

Table 1. Analyzed bioactive peptides and their amino acid sequences, relative molecular masses, M_r, p*I*s and charge at pH 6.5

Peptide	Amino acid sequence	M_r	p*I*	Charge at pH 6.5
Angiotensin I	DRVYIHPFHL	1296.5	7.91	+
Oxytocin	CYIQNCPLG	1007.19	7.70	+
Bradykinin	RPPGFSPFR	1060.2	12.40	++
LHRH	Pyr-HWSYGLRPG	1182.3	7.30	+
Met-enk	YGGFM	573.7	5.93	−

A HP8452 diode array spectrophotometer and a quartz crystal cell were used to obtain the UV spectra. Particle size was verified by SEM and TEM. AuNPs in aqueous dispersion were imaged in a Phillips CM200 Transmission Electron Microscope after placing drops of dispersion onto gold grids, formwar of 400 mesh (SPI, PA, USA), and allowing the liquid to dry in air at room temperature. The morphology of the AuNP-coated capillary column was observed with a field emission scanning electron microscope, Zeiss DSM982 GEMINI SEM with a Field Emission Gun (FEG), operated at 3 kV.

2.3 Synthesis of AuNPs

AuNPs were synthesized by a modification of the Turkevich method [26]. In brief, 4.5 mg of gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$) was dissolved in 45 mL of MilliQ water, and 10 mL of this solution was brought to boil in a round-bottomed flask under stirring. A 1.6 mL portion of a 1% sodium citrate solution was added to the solution using a stirrer. The solution turned red after 5 min, and the intensity of the color increased with time. Boiling was maintained for 20 min after which the heat was removed and stirring was continued for 15 min. Then, 8 mL of the nanoparticle suspension was centrifuged in 1.5 mL Eppendorf tubes at 7000 rpm (4500 × g) during 45 min to remove excess citrate, the supernatant was removed, and the remaining NPs were redispersed in 4 mL of MilliQ water.

2.4 Column preparation

Before coating, the untreated capillary was rinsed with 1 M NaOH for 30 min, followed by water for 30 min, and dried with nitrogen gas (N_2). Subsequently, the capillary was coated by rinsing for 30 min with HCl:MeOH (1:1), flushed with methanol for additional 30 min and dried with N_2. APTES 10% v/v solution in ethanol passed through the fused-silica capillary under pressure for 15 min, then the capillary was rinsed extensively with ethanol and dried in N_2 stream. Finally, the AuNPs suspension was wringed through the APTES-modified capillary for 2 h and washed with pure water.

2.5 Peptide solutions and running conditions

Stock solutions of peptides (1 mg/mL) were prepared by dissolving each synthetic peptide in water, fractionated in aliquots, and frozen at −20°C. Standard solutions were daily prepared by appropriate dilution (1:10) with water. A tryptic

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digest of HSA was prepared as follows: 20 mL of the protein solution (200 mg/mL) was added to 1 mL of 10 mM of potassium phosphate, pH 8.0. Enzyme/substrate ratio was 1:50 m:m and incubation was carried out at 37°C overnight.

The BGE consisted of 20 mM potassium phosphate, pH 6.5, unless otherwise indicated. Peptide solutions were introduced in 5 s at 0.5 psi, and the separation voltage was 12 kV. Before daily work, the capillary columns were washed with BGE for 10 min. After each run, the modified capillaries were washed with BGE for 5 min, and finally were flushed with water at the end of the day.

2.6 UV determinations

About 20 μL of each peptide aqueous solution (0.1 mg/mL) was mixed with 880 μL of BGE and 100 μL of AuNPs suspension in Eppendorf tubes. Adequate blank solution (without peptides) was also prepared.

3 Results and discussion

3.1 Characterization of the AuNP-modified capillary

Figure 1 corresponds to TEM and UV–visible spectroscopy of the synthesized AuNPs dispersed in water. The TEM micrograph shows that the dispersed phase of AuNPs consisted of spherical particles with the size range of 17–20 nm (Fig. 1, inset). The absorption spectra (Fig. 1) show the plasmon resonance absorption maximum in the range of 520–530 nm ($\lambda_{max} = 525$ nm), which further confirms their nanometer-scale size.

Previous reports [27, 28] dealing with the immobilization of AuNPs on the capillary wall required heating or acid treatment to increase the efficiency of separation. On the contrary, this method is simple and showed high stability due to the covalent binding of nanoparticles through APTES derivatization. SEM images (Fig. 2) reveal the morphology and degree of order of the AuNP-coated surface (Fig. 2A and B). A dense and uniform distribution of nanoparticles on the gold substrate can be observed. The mean diameter is conserved, consistent with a monolayer of AuNPs on the inner surface of the capillary.

The modified capillary showed lower absorbance in comparison with the bare one, as the AuNPs attached to the wall of the capillary in the optical path increase the baseline.

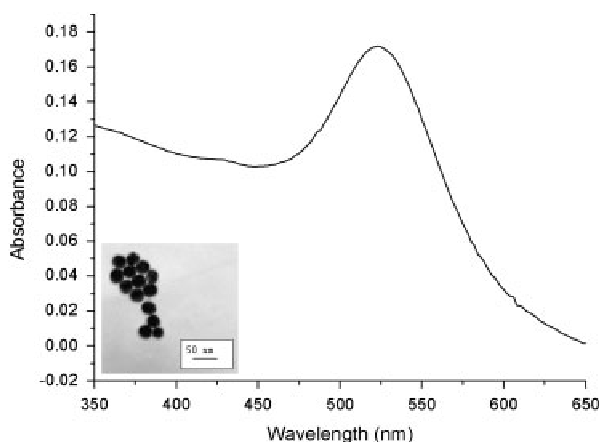

Figure 1. Absorbance spectra in water of AuNPs and TEM micrograph (100.000 ×) (inset).

3.2 UV determinations

The dispersed AuNPs show the typical strong absorption band near 530 nm due to the surface plasmon vibrations in the AuNPs [29]. The addition of the different peptides to AuNPs dispersions turned the color to blue (Fig. 3). The absorption bands were broadened and red shifted (around 700 nm) due to nanoparticle aggregation [30]. This change in the color of the AuNP suspensions occurred only with peptides containing arginine in their structure, A, B, and L. The variation in the optical properties in the AuNPs solutions is demonstrated in the inset of Fig. 3, which shows a picture of the Eppendorf tubes containing different solutions.

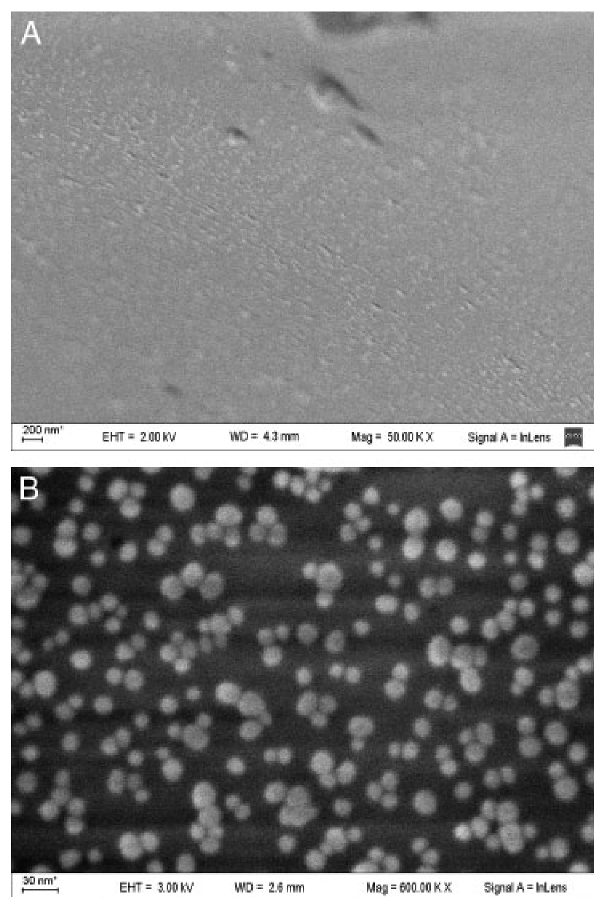

Figure 2. SEM images (magnification 50 Kx (A) and 600 Kx (B)) of AuNP-coated capillaries, AuNPs mean size distribution 15 ± 5 nm.

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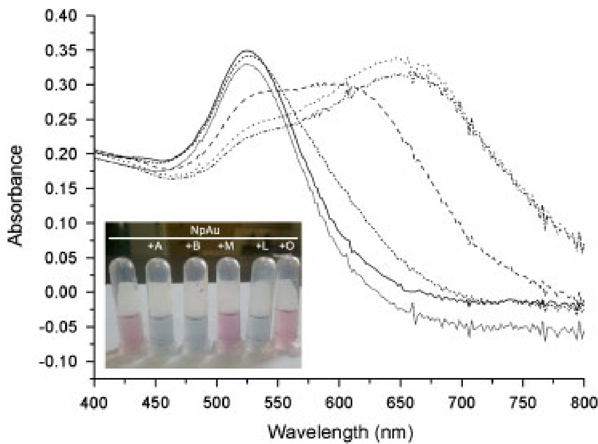

Figure 3. Absorption spectra of suspension of AuNPs, after addition of 20 μL peptide aqueous solution (0.1 mg/mL). Inset: Picture of Eppendorf tubes containing suspension of AuNPs, after addition of 20 μL peptide aqueous solution (0.1 mg/mL).

3.3 Selection of running condition

To illustrate the effects of the capillary surface modification on the chromatographic properties, a mixture of five peptides were studied: A, B, O, M and L. Dimethylformamide was used as an unretained dead volume marker since it is not retained on AuNP-coated capillaries.

The efficiency of phosphate buffer was previously reported [24, 25, 31]. For this reason, we proceed to optimize the system by varying the BGE pH (range 4.0–8.0), voltage, and injection times (not shown). The normalized capacity factor (K_s) of the modified system [32] duplicates the observed for the fused silica (results not shown). It was stable during the optimization process; therefore, this modification is stable and can be applied for several separation purposes.

Figure 4A shows the electrochromatograms representing the separations of A, B, O, M, and L at pH ranging from 5.0 to 8.0 on the AuNP-coated capillary. When the pH is acidic (pH 4) the peptide is protonated, positively charged, and totally retained (not shown), as the pH increased the running time diminished, as is demonstrated in Fig. 4B). The best separation, best resolution, and shorter time were obtained with pH 6.5, nearly biological pH. The migration time of peptide above pH 6.5 is independent on the solution pH, probably due to covalent bonding between free amine groups and supported AuNPs [32].

3.4 Separation in AuNP-modified capillaries

Once the running condition was obtained, the peptide mix was tested on CZE and AuNP-coated capillary as shown in Fig. 5A and B. Although for the AuNP-coated capillary, the whole electrophoretic run was twice longer, better resolution and peak shape were obtained. In fact, the theoretical plate numbers (N) for the studied solutes were duplicated: 11622 (CZE) and 23066 (OTCEC).

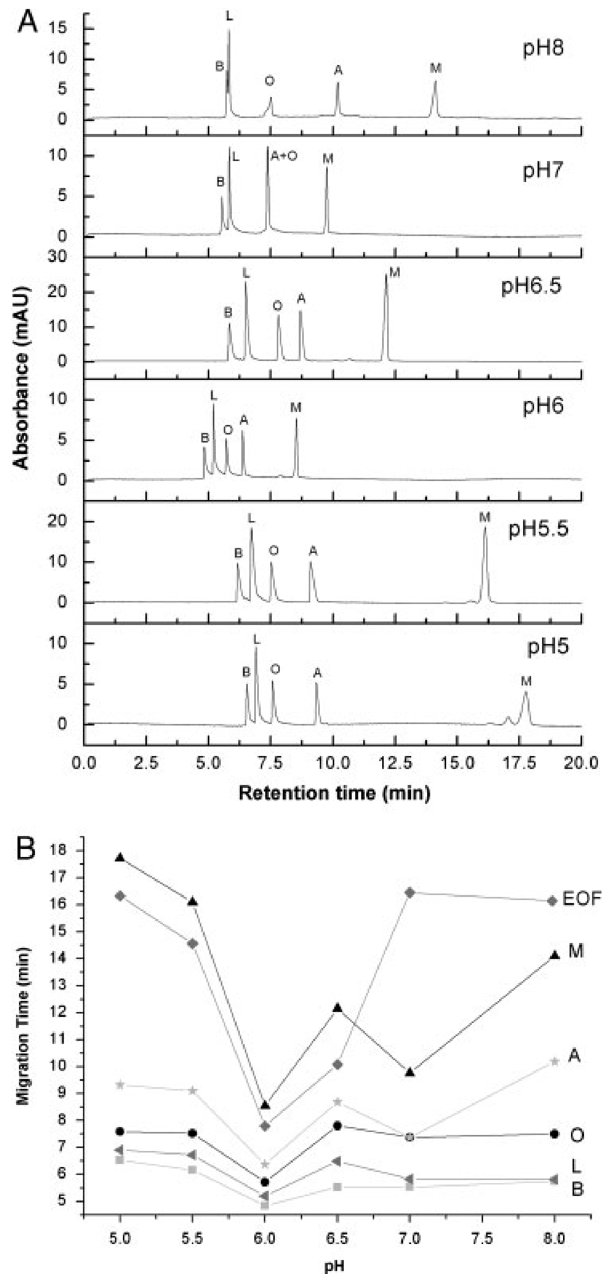

Figure 4. (A) Separation of B, A, L, O and M at different pH (5.0–8.0), 50 μg/mL. BGE, 20 mM potassium phosphate; 12 kV; sample introduction time, 5 s at 0.5 psi; separation capillary, 75 μm id × 41.2 cm in length (31 cm to the detector). (B) Migration time versus BGE pH in the AuNPs-modified column. BGE, 20 mM AuNPs phosphate, pH 5.0–8.0; sample introduction time, 5 s at 0.5 psi; 12 kV.

We performed derivatization of three different batches, three capillaries each, using the same technique. In these experiments, high reproducibility of electrophoretic mobilities was seen. Moreover, migration times and separation were more reproducible than with bare-fused-silica capillaries. Also, the AuNP-coated capillaries probed to be stable over time, as they were prepared four months prior to use and reused 900 times.

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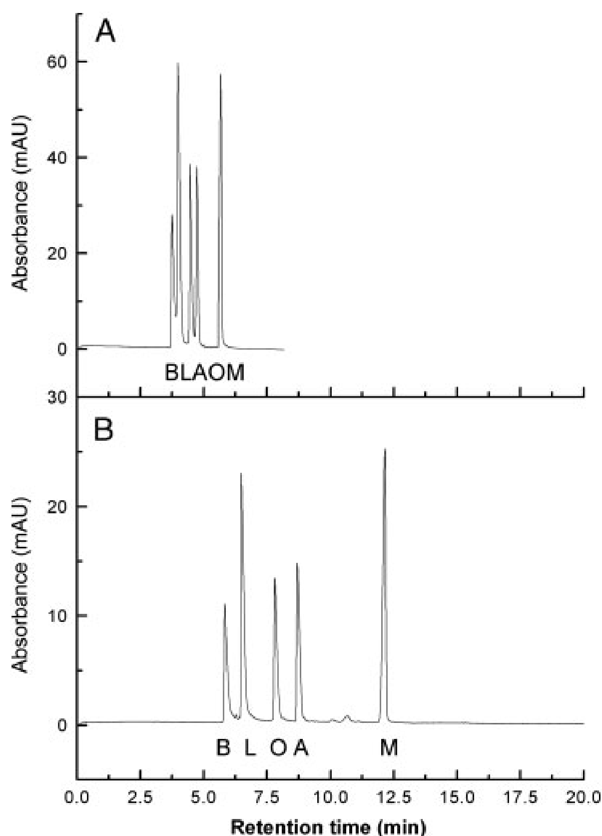

Figure 5. Separation of B, A, L, O and M, 50 μg/mL. BGE, 20 mM potassium phosphate, pH 6.5; 12 kV; sample introduction time, 5 s at 0.5 psi; separation capillary, 75 μm id × 41.2 cm in length (31 cm to the detector). (A) Bare-FS capillary and (B) AuNP-coated capillary.

A significant change in sequence of peptide separation was observed in the modified capillary. A, B, and LHRH (L) reduced their electrophoretic mobility, while O increased it. Met-enk did not suffer any modification on its electrophoretic mobility (Table 2).

These results are consistent with the UV–visible analysis. Biomolecules containing thiol (SH) or amino (NH_2) groups can be adsorbed spontaneously onto gold surfaces to generate well-organized, self-assembled monolayers. Then, AuNPs have exceptionally high affinity for peptides due to hydrophobic interaction and covalent conjugation, which is believed to occur mainly by direct attachment of cysteine, lysine, and arginine residues of peptides to gold surface. (Table 1).

Peptides A, B, and L contain arginine and although O has two cysteines in the molecule, they form a disulfide bridge and are not available to interact with the AuNPs.

3.5 Separation of tryptic digest of HSA

A complex mixture of tryptic peptide fragments of HSA was analyzed in both the bare FS and the AuNP-coated capillaries (Fig. 6A and B). The runs were performed at different pHs in order to improve peak resolution (not shown). As in mixture peptides assay, the better electrophoretic profile was observed using BGE at pH 6.5. The coating of the inner capillary surface resulted in a

Table 2. Effective electrophoretic mobilities, CV% and significant differences of the analyze peptide

	CZE		OT-CEC		Differences between capillaries[b]
	Effetive electrophoretic mobilities[a]	CV%	Effetive electrophoretic mobilities[a]	CV%	
Bradykinin	8.02	2.42	7.72	1.68	Yes
LHRH	6.35	2.47	6.08	1.59	Yes
Oxytocin	2.23	6.70	3.43	0.76	Yes
Angiotensin I	3.47	6.77	2.05	0.84	Yes
Met-enkephalin	−1.43	7.27	−1.31	2.95	No

a) μ_{eff} (10^{-3} $cm^2\,V^{-1}\,min^{-1}$).
b) With 5% of confidence level.

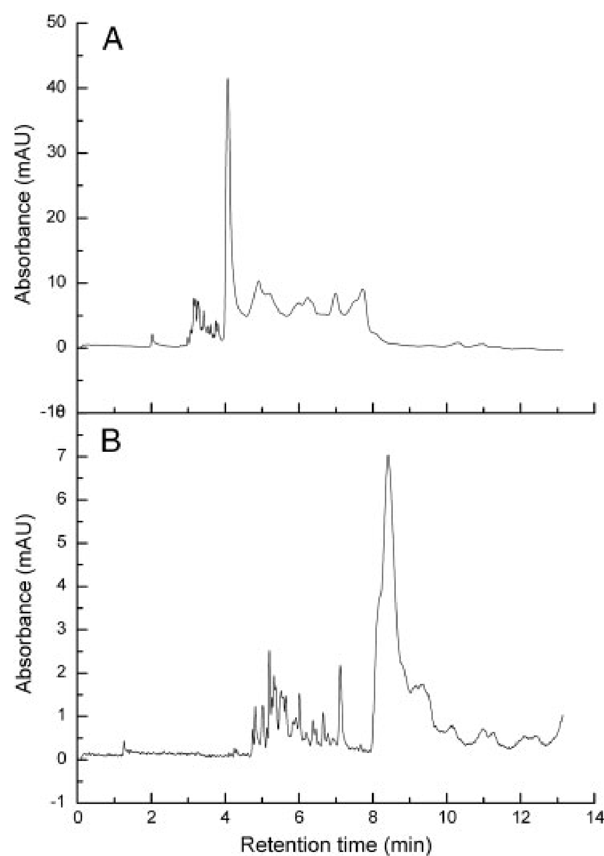

Figure 6. Separation of an HSA tryptic digest. BGE, 20 mM potassium phosphate, pH 6.5; sample introduction time, 5 s at 0.5 psi; 12 kV; separation capillary, 75 μm id × 41.2 cm in length (31 cm to the detector), (A) bare-FS capillary and (B) AuNP-coated capillary.

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broader separation window. As a consequence, resolution was improved for some fragments at the expense of longer separation time. Finally, the analytical criteria adopted in this work may be applied for the analysis of samples from biological source as well as peptide formulations to be used in biological systems.

4 Concluding remarks

In this paper, we have demonstrated a new method for the separation of a mixture of synthetic peptides with a high degree of selectivity and also to achieve a tryptic profile suitable for further studies (for example, elucidation of the structure by engaging in a mass spectrometer).

The immobilization of AuNPs on the capillary wall was performed in a very simple way without requiring heating and in a very short time. The method resulted in a very stable coverage since the capillary was reused for about 900 times. The chromatographic properties of AuNP-coated capillaries were investigated through variation of the organic modifier percentage in buffer, buffer pH, and separation voltage. Effective separations of the test mixture were obtained on the AuNP-coated capillaries. This study also emphasizes that the resolving power of separation depends on the interaction between peptides and AuNPs, and could significantly alter the electrophoretic mobility.

The AuNP-modified capillary showed a higher background in comparison to the bare capillary. This effect is attributed to the absorption on the nanoparticles and could be improved by the change in either size or surface concentration of nanoparticles. We are working on these experimental aspects to improve the sensitivity of the method

Finally, the analytical criteria adopted in this work may be applied for the analysis of samples from biological source as well as peptide formulations to be used in biological systems.

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The authors have declared no conflict of interest.

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