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Analysis of intact proteins with capillary zone electrophoresis coupled to mass spectromery using uncoated and coated capillaries

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ABSTRACT

Although, in general, the application of coated capillaries is recommended for the separation of intact proteins, bare silica capillary is still the most often used capillary due to its simplicity and cheapness. In this work, the performance of bare fused silica capillary for intact protein analysis was compared to that of different (dynamically coated polybrene (PB) and permanently coated linear polyacrylamide (LPA)) coated capillaries using capillary zone electrophoresis - mass spectrometry (CZE-MS). In cases where low pH (pH=1.8) was used in bare silica capillaries, good precision (0.56-0.78 RSD% and 1.7-6.5 RSD% for migration times and peak areas, respectively), minimal adsorption and separation efficiency (N= 27 000/m - 322 000/m) similar to or even better than those obtained with the coated capillaries (created by an intricate multi-step process) was achieved. The PB and the LPA capillaries demonstrated their slightly better resolving power in terms of separating the different forms/variants of the same protein (e.g., hemoglobin subunits). Among the studied capillaries the one with LPA coating showed the most stable separations in the long term (n=25: 0.18-0.49 RSD% and 3.1-4.9 RSD% for migration times and peak areas, respectively). For the separation of a few proteins or even a larger number of proteins in biological samples (e.g., snake venom) the application of the simple and cheap bare fused silica capillary can be considered as an efficient choice.

1. Introduction

Proteins and protein complexes perform vital roles in the cells, from transportation of metabolites and assisting biochemical reactions to regulating gene expression and ensuring structural stability. For understanding the function and structure of proteins, their efficient analysis is essential, which requires the combination of high-resolution separation methods and high-performance mass spectrometry detection [1–3].

In most proteomics studies the bottom-up approach is employed, where the proteins of interest are digested into peptides prior to their MS determination. However, the advantages associated with the high-throughput performance and considerably easier execution of this approach can fail in providing significant information on post-translational modification (PTM) forms and the structure of the assemblies following proteolysis [4]. In contrast, top-down mass spectrometry (TDMS) can provide better insights into the proteoform variability and complexity since proteins are introduced into the MS system in their intact form without any chemical or enzymatic digestion or treatment [5–9].

The most common analytical method for intact protein analysis is liquid chromatography (LC) including reversed-phase liquid chromatography (RPLC), hydrophobic interaction LC and ion exchange (IEX) chromatography [10]. Besides chromatography, electrophoretic techniques have received much attention for intact protein analysis. SDS polyacrylamide gel electrophoresis (SDS-PAGE) [10] is widely used for the separation of proteins, under denaturing conditions. Capillary electrophoresis (CE) has an increasing popularity in the separation science of intact macromolecules. Several appealing aspects of CE over traditional slab gel methods are the faster analysis, high separation efficiency, low consumption of samples and the possibility for on-line detection [1,11]. Even though several CE modes (CZE, CIEF, CGE) can be employed for protein analysis, only CZE [6,12–16] offers the simple applicability with MS compatible electrolytes. However, analyte adsorption in the separation capillary remains a major concern [17–19], which may result in impaired separation efficiencies, fluctuating electroosmotic flow (EOF) and deviations of migration times. Therefore, several

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techniques have emerged to minimize the analyte-wall interactions (e.g., ionic or hydrophobic), including the use of "extreme" pH background electrolytes (BGE) [20] and additives [20–22] or the modification/coating of the inner surface of the capillary [23–32].

The last 3 decades saw an immense increase in works reporting about new capillary coatings for the separation of proteins [17–19,23–24]. In addition, many reviews [6,9,17,19,30,31] discussed and evaluated these results and applications. These works clearly prefer the use of coated capillaries for intact protein separation to prevent the adsorption effects and to obtain constant migration times. However, a considerable part, 45% of the papers on CE-MS (data obtained from Ref. [9], which reviewed 94 papers) used bare fused silica capillaries and the most often applied dynamic and static coatings were the PB (14%) including the three-layer SMIL coating, as well [33] and the LPA (9%). In several papers the comparison of the different capillaries was made in an "unfair" way. These studies typically employed the same separation conditions (which was ideal only for the coated capillary) for both coated and uncoated capillaries – neglecting the fact that the parameters (mainly the pH) may not be ideal for the bare silica capillary [22,25,26,31]. Indeed, the bare silica capillary is well applicable for intact protein separation only in very low or very high pH conditions [34].

The aim of the present study was to compare the separation efficiencies achievable with the two most often applied coated capillaries and the bare fused silica capillary for intact protein analysis using the optimal conditions for each capillary. In this study we applied BGEs and conditioning solutions which are compatible with MS detection. For the comparison studies high protein content snake venom sample was used, as well.

2. Materials and methods

2.1. Chemicals and materials

Formic acid (FA), acetic acid, ammonia, ammonium acetate (NH_4Ac), acetonitrile, isopropylalcohol (IPA), 3-(trimethoxysilyl)propyl methacrylate, sodium hydroxide, hydrochloric acid, methanol were obtained from VWR (Radnor, PA, USA). BGE additive hexadimethrine bromide (polybrene, PB), and proteins such as lysozyme from chicken, human hemoglobin, myoglobin from equine skeletal muscle, human serum albumin (HSA), human thyroglobulin lyophilized powders of analytical grade were from Sigma Aldrich (St. Louis, MO, USA). Human insulin solution (Humulin R) with the 3.5 mg/mL concentration was purchased from Lilly (France). The theoretical molecular masses and the isoelectric points of the studied protein standards were provided in the Table SM-2. Tris-HCl, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulfate (APS) and acrylamide were obtained from Sigma. Dextran sulfate (DS) was obtained from Merck Millipore (Darmstadt, Germany). Snake venom sample (Naja melanoleuca, #304) was received from Dr. Vladimir Petrilla, Department of Biology and Physiology, University of Veterinary Medicine and Pharmacy, Kosice, Slovakia) Deionized water (Millipore Synergy UV) was used for making the aqueous solutions.

BGE solutions were degassed for 5 minutes in an ultrasonic bath. 0.45 µm pore size membrane filters were used to filter all BGE solutions. The stock solutions were stored at +4°C. The rinsing procedure of the bare silica capillary before its first use comprised of flushing with 1 M NaOH for 20 min, deionized water for 5 min, and finally with running buffer solution for 20 min. Polymerization solution used for LPA capillary coating procedure contained 1 mL degassed 4 % w/w acrylamide solution, 1 µL TEMED and 10 µL 10 % w/w APS solution.

2.2. Instrumentation and operating conditions

A 7100 model CE instrument (Agilent, Waldbronn, Germany) equipped with UV and MS (maXis II UHR ESI-QTOF MS instrument, Bruker, Bremen, Germany) detectors were used to perform the measurements. UV detection was conducted at 200 nm by on-capillary photometric measurement. Samples were injected hydrodynamically (50 mbar, 2 s) at the anodic end. External pressure (max. 7 bar) could be obtained from a nitrogen cylinder. Data acquisition and processing was carried out by means of OpenLAB CDS Chemstation (Agilent) software.

In terms of MS detection, electrospray was formed by CE-ESI sprayer (G1607B) by Agilent which enabled online coupling with the CE instrument. A 1260 Infinity II isocratic pump (Agilent) provided the transfer of the sheath liquid. The measurements were supervised with the help of otofControl software version 4.1 (build: 3.5, Bruker) and acquired data was processed by Compass DataAnalysis version 4.4 (build: 200.55.2969). 6 Hz spectra rate was used.

Sheath liquid solution was transported at a flow rate of 7 µL/min and contained IPA:water = 1:1 + 0.1 % v/v FA. MS operating parameters were: nebulization pressure: 0.5 bar, temperature of dry gas: 220°C at 8.0 L/min flow rate, capillary voltage: 4500 V, end plate offset: -500 V. MS scans were over the 500-2400 m/z range for protein samples. ESI-MS Tuning mix solution (Agilent) was used for internal calibration.

Unless otherwise stated, the length of fused silica capillaries (50 µm I.D. and 370 µm O.D. (effective length (L_{eff}): 57 cm for UV) (Polymicro, Phoenix, AZ, USA)) were 65 cm and 85 cm for analyses with UV and MS detecion, respectively. For analyses with uncoated capillaries 1 M FA (pH=1.8), 50 mM NH_4Ac (pH= 6.8 and pH= 9.6) and 100 mM NH_4OH (pH=10.8) were used as BGE; 50 mM FA (pH=2.6), and 1 M acetic acid (pH=2.5) were used for the LPA and PB coated capillaries, respectively. Postconditioning procedures for the different capillaries are described later. For the separations in polybrene coated capillaries reversed polarity of the separation voltage had to be applied (-30 kV). The current during the electrophoresis with MS detection was not higher than 20 μA.

2.3. Capillary coating

PB dynamic coating procedure was applied as reported elsewhere [32]. Following the deprotonation of silanol groups by using 1 M NaOH for 20 min (1 bar), the fused silica capillary was flushed with water, 10% w/v PB solution and 1 M acetic acid (pH = 2.5) for 20 min, 30 min and 40 min, respectively applying a pressure of 1 bar. After each run the coating was regenerated with the PB solution and BGE for 5 min each. For coating/regeneration procedure the CE capillary was displaced from the ESI chamber. During the CE run the bleeding of polybrene was minimal and did not cause suppression of ionization or considerable background signals (as reported by others [21]). The three-layer (PB-DS-PB, SMIL: successive multiple ionic-polymer layer) coating was prepared according to ref. [33].

The preparation of the LPA static coating was based on the description by Hjertén [25,35]. A new capillary was flushed with acetone (3 min, 6 bar), 1 M NaOH (60°C, 30 min, 5 bar), deionized water (1 min, 6 bar), 1 M HCl (30 min, 5 bar) and again deionized water (1 min, 6 bar). Then capillary was dried using nitrogen flow (30 min, 6 bar and 60 min, 2 bar). Afterwards, the capillary was filled with the silane mixture (25°C, 5 min, 3 bar) which contained 3-(trimethoxysilyl)propyl methacrylate (Sigma) and acetonitrile at 1:1 ratio, and kept immersed in the reagent overnight. The following day, unbound silane was removed by acetone (1 min, 2 bar), then the capillary was dried with nitrogen flow (60 min, 2 bar), and finally flushed with LPA polymerization solution and kept at

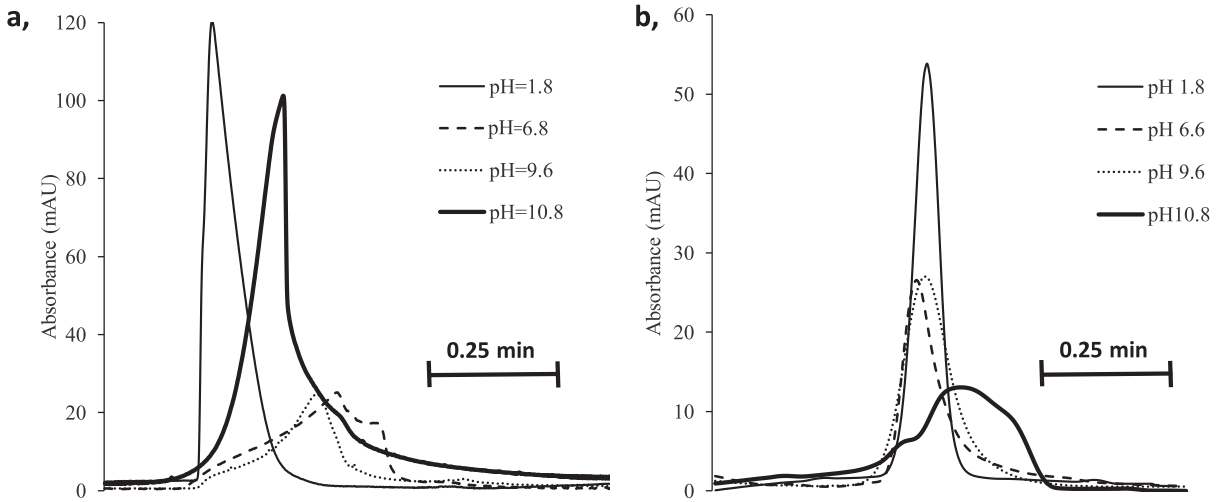

Fig. 1. Peaks of human insulin (**a**) and human serum albumin (**b**) obtained with capillary electrophoresis at different pH values (the peaks are shown as sections of the electropherograms). Conditions: capillary: 31 cm x 50 µm id., separation voltage: 20 kV, injection: 50 mbar x 2 s, sample matrix: deionized water, BGE: 1 M FA (pH 1.8), 50 mM NH_4Ac (pH 6.8 and 9.6), 100 mM NH_4OH (pH 10.8), postconditioning: wash with 1 M NaOH (5 min, 1 bar), water (2 min, 1 bar) and BGE (5 min, 1 bar), UV detection at 200 nm. The concentration of human insulin and HSA was 0.5 mg/mL

50°C for 60 min and then at room temperature overnight. The next day, the capillary was rinsed with deionized water to eliminate the excess of polymer solution. For storage, the coated capillary was filled with deionized water, and the capillary ends were kept immersed in deionized water. The preconditioning for each run involved a flushing step with running buffer for 5 min (1 bar). No organic solvent or solutions with pH>8 and pH<2 were allowed to be used.

3. Results and discussions

3.1. Separation in bare fused silica capillary

In order to find suitable conditions for the separation of intact proteins in a bare silica capillary, five different proteins were analysed using BGEs with „extreme" pH values (pH=1.8 and 10.8) and pH more commonly used for CE separations (pH= 6.8 and 9.6). At very low pH the silanol groups are completely protonated leading to a neutral charge of the silica surface, while the proteins (far below their pI) possess a large net positive charge. On the other hand, the very high pH of the BGE results in the complete deprotonation of the silanol groups, and thus a negatively charged capillary surface, but also the proteins have a big net negative charge. No electrostatic or hydrophobic interactions can occur between the proteins and the channel wall in either case, hence minimal adsorption and narrow peak shape was expected. Indeed, the narrowest peak was obtained in the case of BGE of pH=1.8 for insulin and HSA (Fig. 1), while in "moderate" pH range, wider and therefore shorter and tailed peaks were attained as a result of electrostatic interactions. The strength of these adsorption effects largely depends on the pI (5.35 and 4.7 for insulin and albumin, respectively) and the structure of the protein. However, the very low or very high pH electrolytes may cause the denaturation and precipitation of proteins. The electrophoretic migration of the protein precipitated in the capillary leads to a non-symmetrical progression of the sample zone, resulting in smaller and strongly distorted peak shapes. The precipitation of the protein can be the reason for the non-symmetrical and small signal obtained in the case of BGE of pH=10.8. Fig. 1. shows that a good separation efficiency for both smaller (insulin, M_r: 5.7 kDa) and larger (HSA, M_r: 66 kDa) proteins can be achieved only in low pH medium. (For instance the theoretical plate numbers are at least 10 times smaller above pH 3 in the case of insulin.) Ref. 20 demonstrated the bare fused silica measurements of proteins employing several acidic buffer solutions, with different concentrations. It has been mentioned that the superior efficiency and resolution was provided by formic acid at 1 M concentration (pH=1.8) compared to phosphate, acetic and propionic acids [20]. At pH=1.8 the peak shape of HSA is not only narrow but symmetrical, while insulin shows larger peak distortion. This right-angled triangle shape is reminiscent of electrodispersion phenomenon, which is a usual consequence of the large difference in mobilities between the analyte and the electrolyte ion. Electrodispersion is well-known for small analytes [34] but not described for proteins. As a fact, at very low pH insulin (being a very small protein) possesses a considerably large charge-to-size ratio. Nevertheless, both electrodispersion and overloading effects can be largely suppressed by analysing diluted samples. Using BGEs with "moderate" pH (close to the pI values of the proteins), a part of the protein is adsorbed to the surface modifying the capillary surface, which can alter the EOF and can enforce the adsorption of components from the subsequently injected samples.

One might think that the larger the protein, the stronger the adsorptive forces (the larger protein includes more groups capable of making more interactions); however, quite narrow peaks can be obtained even for huge proteins (e.g., thyroglobulin, M_r: 880 kDa, Fig. SM-1 and Fig. SM-4). At pH=1.8 the peak shapes of small molecules and larger proteins are very similar after a short separation length and those are widened with the increase of the separation length to a similar extent (Fig. SM-4). Although the adsorption seems minimal at pH=1.8, the complete renewing of the capillary surface after each electrophoretic run is advised. The comparison of the results obtained with and without postconditioning exposed the substantial need for washing the capillary with strong base (1 M NaOH or 1 M NH_4OH) and/or strong acid (1 M HCl or FA). The precision (n=20, thyroglobulin) for migration times were 5.3 RSD% and 0.8 RSD%; for peak areas were 6.3 RSD% and 2.7 RSD% with no postconditioning and with postconditioning, respectively. For the separation of several intact proteins a capillary-postconditioning protocol including a successive wash with $NaOH/NH_4OH$ (5 min), water (2 min) and BGE (5 min) is suggested. When using a BGE with moderate pH for the separation of proteins, a considerable shift in migration times and broadening of the peaks could not be avoided even when the proposed postconditioning was applied (Fig. SM-2 and Fig. SM-3). In the case of such BGEs, often the

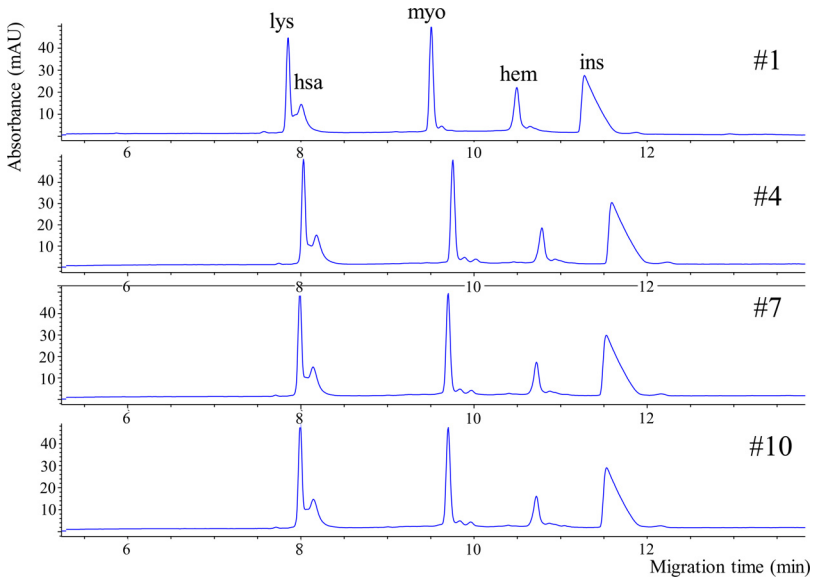

Fig. 2. The separation of 5 proteins in bare fused silica capillary. The repeated separations were represented by the 1st, 4th, 7th and 10th runs. Conditions: capillary: 65 cm x 50 µm id., BGE: 1 M FA (pH 1.8), postconditioning: wash with 1 M NaOH (12 min, 1 bar), acetone (6 min, 1 bar) and BGE (20 min, 1 bar). Sample: 0.5 mg/mL lysozyme (lys), human serum albumin (hsa), insulin (ins) and 1 mg/mL hemoglobin (hem), myoglobin (myo). The other parameters were the same as stated in Fig. 1.

Table 1
Precision data (RSD% of migration times and peak areas) obtained in different capillaries. The analysis conditions were the same as stated in Fig. 2.

	bare fused silica			dynamic coating (PB)			static coating (LPA)		
Protein	RSD% (t)	RSD%(area)	N (/m)	RSD% (t)	RSD%(area)	N (/m)	RSD% (t)	RSD%(area)	N (/m)
lysozym	0.56	2.76	177090	0.59	4.52	44205	0.18	3.16	84062
HSA	0.54	3.44	47706	0.69	3.45	5381	0.20	3.38	17047
myoglobin	0.64	1.66	321771	0.62	2.36	39006	0.31	3.96	75232
hemoglobin	0.70	6.54	193451	0.75	12.7	36387	0.43	4.33	117988
insulin	0.78	2.91	26676	0.69	5.54	18971	0.49	4.86	7127

proteins might completely adsorb and their peaks can not be observed in the electropherogram. The dependence of the adsorption effects on the pH of the BGE and pI of the proteins are straightforward [6,17,19]. Performing the separation in a BGE of pH=6.8 or pH=9.6 the net charge of lysozyme is positive (pI=11.35), suggesting a strong electrostatic attraction to the negatively charged silica surface, which resulted in lysozyme disappearing from the electropherogram (Fig. SM-2 and Fig. SM-3).

With the application of a low pH BGE (1 M FA) and the postconditioning procedure described above, efficient and well reproducible separations of a mixture of proteins could be achieved even in a bare silica capillary (Fig. 2). For 5 proteins including lysozyme, human serum albumin, myoglobin, hemoglobin and insulin, the precision data were better than 1 RSD% and 4 RSD% (except hemoglobin) for the migration times and peak areas, respectively (Table 1). It should be noted that the migration times of the first run of the repetitions often remarkably differs from the subsequent runs, therefore the first run of each day should be considered a kind of preconditioning/pretreating step (the first run was not included to the calculation of RSD%). (The monotonous decrease of peak areas of hemoglobin was probably caused by the change of this protein in the sample solution and not its adsorption.)

The plate numbers were in a wide range from 27 000/m to 322 000/m (Table 1). Insulin has the broadest peak. However, when insulin was largely diluted (0.027 mg/mL), efficiency could be increased to 243 000/m as the peak distorsion was suppressed. Stutz et al [20] reported up to 165 000/m for carbonic anhydrase (the plate numbers for other proteins of the sample were much smaller) with 1 M formic acid.

Switching from UV to MS detection two differences could be expected: (1) the variability of detection response factors (sensitivity) for the components and (2) the on-capillary detection (UV) provides slightly better resolution compared to off-capillary detection (MS). While all studied proteins have similar UV spectrum, these proteins form different numbers of charge forms (and several adducts, as well) in ESI leading to different detection sensitivities (Fig. 3). For instance, albumin is present in ~ 45 different charge forms (these different forms and their adducts cannot be distinguished within the charge envelope), but lysozyme shows only 4 different charged forms. The larger the number of charged forms, the smaller the overall signal intensity for that particular electrophoretic peak. The separation efficiency in the case of MS detection is slightly (<5%) reduced due to the laminar flow generated by the small vacuum at the outlet of the CE capillary in the ESI interface ("siphon effect"). This effect can be attenuated by switching off the ESI nebulization pressure during sample injection and the first few minutes (around 20 s before the first component of interest reaches the end of capillary) of electrophoresis [36]. The use of MS detection provides the possibility to identify the proteins based on the exact mass of the molecule (Table SM-1).

Although in CE the use of time reference marker(s) is often inevitable in order to obtain acceptable RSD% for migration times, in the case of bare fused silica capillary this kind of normalization was not necessary for the BGE with pH=1.8 (0.56-0.78 RSD% and 1.7-6.5 RSD% for migration times and peak areas, respectively).

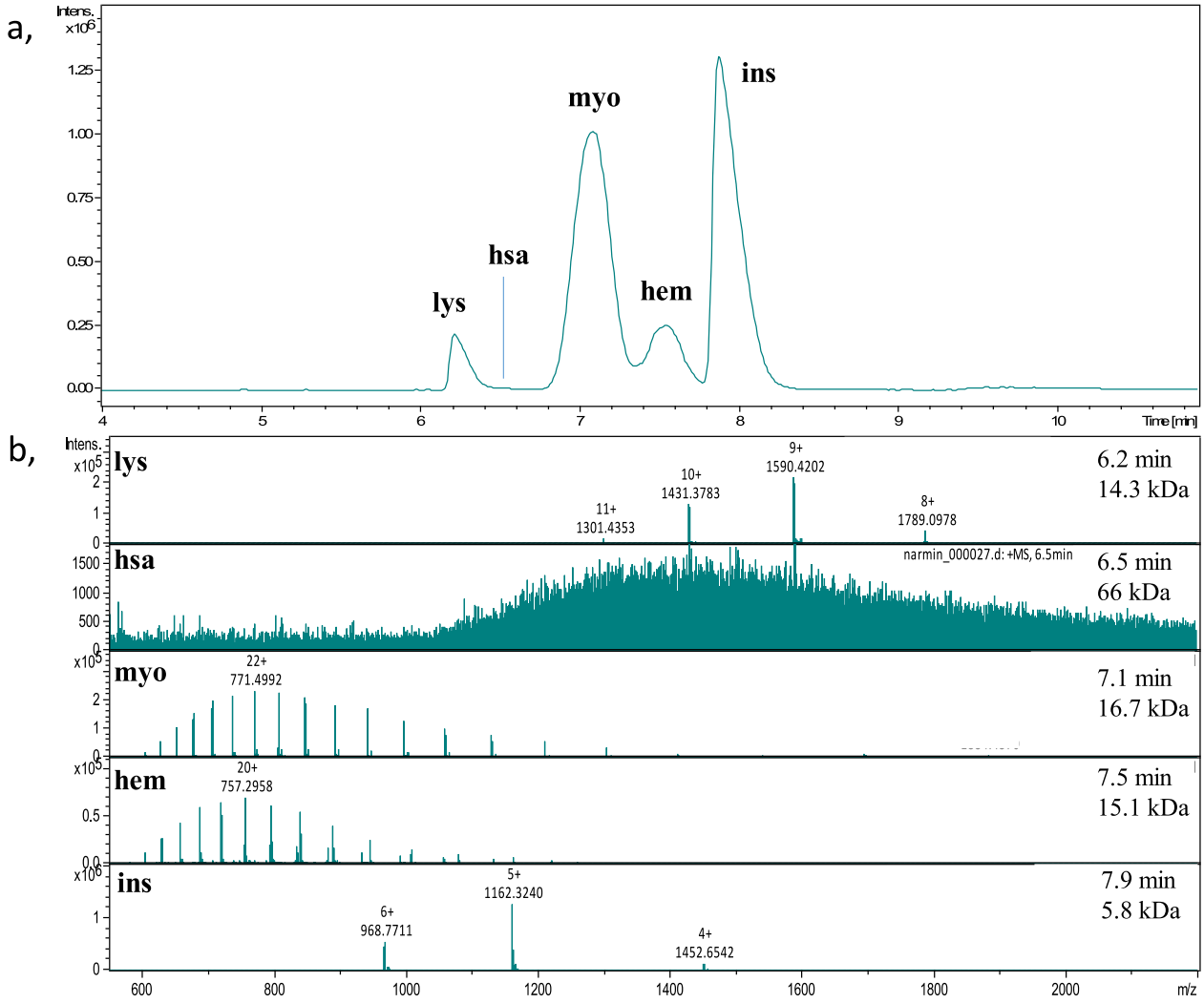

Fig. 3. CZE-MS analysis of a protein mixture. Base peak electropherogram (**a**) and mass spectra of separated proteins (**b**) Conditions: nebulization ESI pressure: 0.5 bar, dry gas temp.: 220°C, spectra rate: 1.5 Hz, m/z range: 500-2400. The other parameters were the same as stated in Fig. 2.

When the separation was conducted at a higher pH then two time reference markers should be applied to achieve proper precision for migration times, but these markers can not help in achieving better than 6 RSD% for peak areas (Fig. SM-2 and Fig. SM-3).

3.2. Separation in coated capillaries

In our work, the dynamically (PB) and permanently (LPA) coated capillaries most often applied for CE-MS were studied. The separation conditions (including MS-compatible BGE and conditioning solutions) for intact proteins were optimized.

The positively charged PB lends a charge to the capillary wall which does not depend on the pH of the BGE thus the anodic EOF remains constant. Since the capillary surface is positively charged it is worth using low pH for the separation of cationic proteins. For PB coating, 1 M acetic acid (pH=2.5) was optimal in good agreement with others [22,32]. In the electropherogram (Fig. 4.b) the proteins appear after the EOF (reversed polarity separation voltage is applied) and in a migration order that is just the opposite of that found with the bare fused silica capillary. The analysis time with PB is longer because the components migrated against the EOF. The larger migration times caused wider peaks, and the separations were similar to or worse than those obtained with non-coated capillary at pH=1.8. For instance, the resolution values between hemoglobin and myoglobin were 4.12 and 3.21 in bare fused silica and PB coated capillary, respectively. In some cases even the different variants of the given protein were revealed. For instance, the different subunits (αa, βa, βa', Mr: 15 125, 15 867, 15 881, respectively) and an unknown variant of hemoglobin were separated (Fig. 5.). The separation of the αa and βa was also reported by others in PB coated capillary [20]. Although the achievable separation efficiency is slightly lower with MS detection (N= 30 000/m) compared to the on-capillary UV detection (N=36 000/m), the same number of variants of hemoglobin could be separated with both detection methods, but the identification of the different forms could only be carried out with MS (Fig. SM-5). Very recently intensive efforts have been made to further improve the SMIL technique and its different versions. With proper modulation of the electroosmotic mobility using polyelectrolyte multilayer coatings very impressive separation efficiencies can be expected for proteins [37]. Not only three but five or even more layer coatings are suggested for the separation of basic proteins in acidic conditions [29]. Our work was limited to studying the one layer PB and three layer SMIL.

The separation of proteins in LPA as a neutral, covalently coated capillary proved to be a succesful strategy [25–31]. The advantages of LPA are that there is no need for the regeneration of the coating, negligible adsorptive interactions with proteins were observed

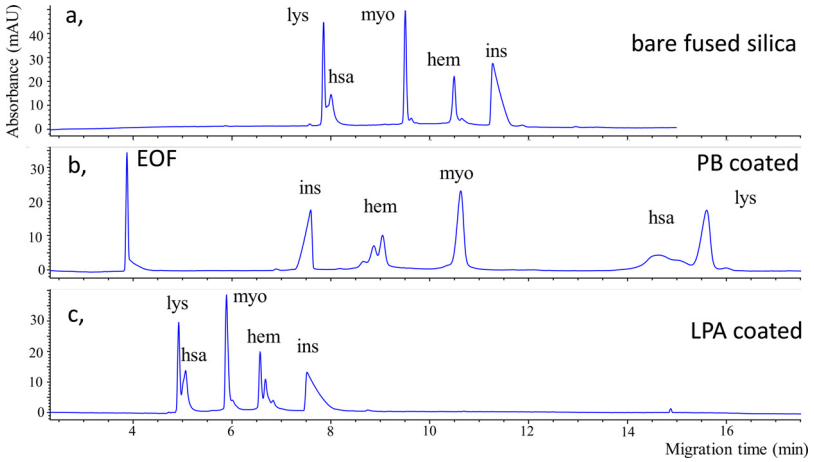

Fig. 4. Separation of a protein mixture in bare fused silica (**a**), polybrene (**b**) and linear polyacrylamide coated (**c**) capillaries. Conditions: **a**: separation voltage: +20 kV, BGE: 1 M FA (pH 1.8), postconditioning: wash with NaOH (12 min, 1 bar), acetone (6 min, 1 bar) and BGE (20 min, 1 bar) **b**: separation voltage: -30 kV, BGE: 1 M acetic acid (pH 2.5), postconditioning: BGE (5 min, 1 bar), preconditioning: polybrene (5 min, 1 bar) and BGE (5 min, 1 bar). **c**: LPA coated capillary, separation voltage: +30 kV, BGE: 50 mM FA (pH 2.6), postconditioning: BGE (5 min, 1 bar). The other parameters were the same as stated in Fig. 2.

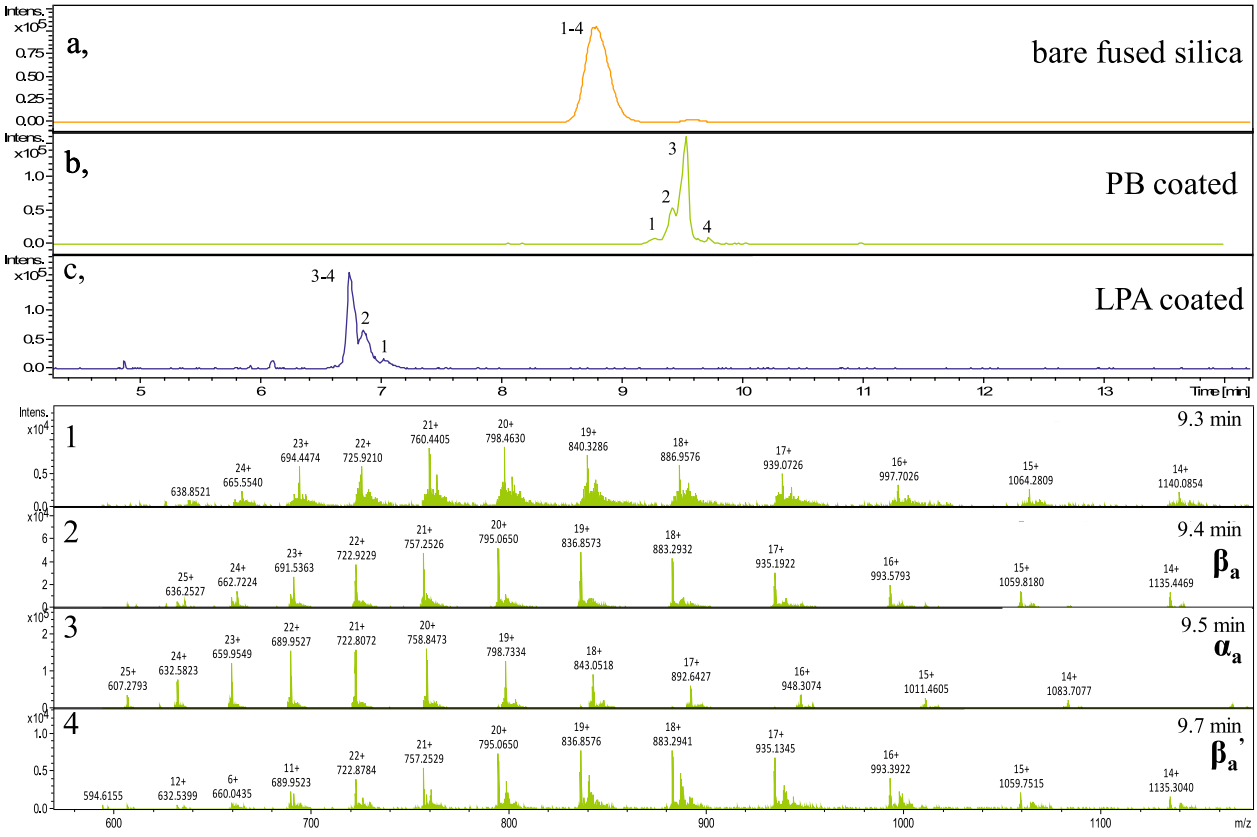

Fig. 5. CZE-MS analyses of hemoglobin in bare fused silica (**a**), polybrene coated (**b**) and LPA coated (**c**) capillaries. The mass spectra of the components separated in polybrene coated capillary (**b**) were shown. Sample: 5 mg/mL hemoglobin. The CZE conditions were same as in Fig. 4 and MS parameters were identical as in Fig. 3.

and the coating can be made in a laboratory of average infrastructure. A notable drawback of LPA is the limited variety of acceptable solvents: it must not be used with electrolytes with pH<2 and pH>8 or with a liquid including organic solvent. EOF is not generated in the neutral LPA capillary, therefore at pH=2.5 similar migration times should be obtained with LPA and bare silica capillary (in the latter capillary very low EOF is expected at this acidic pH). However, in the case of bare fused silica capillary, due to the progressive adsorption of cationic components onto the capillary wall, a gradually increasing, counter directed EOF developed (Fig. 4.a. and c., and also Fig. SM-6.a.-g.). For long-term precision (n=25) good precision data (0.18-0.49 RSD% and 3.2-4.9 RSD% for migration times and peak areas, respectively) were obtained in LPA capillary (Table 1.) even if only the BGE is used for postconditioning. The repetition studies for LPA coated capillaries were performed in a coated capillary which was used for more than 100 separations of proteins. This fact demonstrates the reliability and efficiency of the LPA capillaries. The PB coating offers slightly worse repeatibility (0.59-0.75 RSD% and 2.4-13 RSD% for migration times and peak areas, respectively), although no considerable adsorption

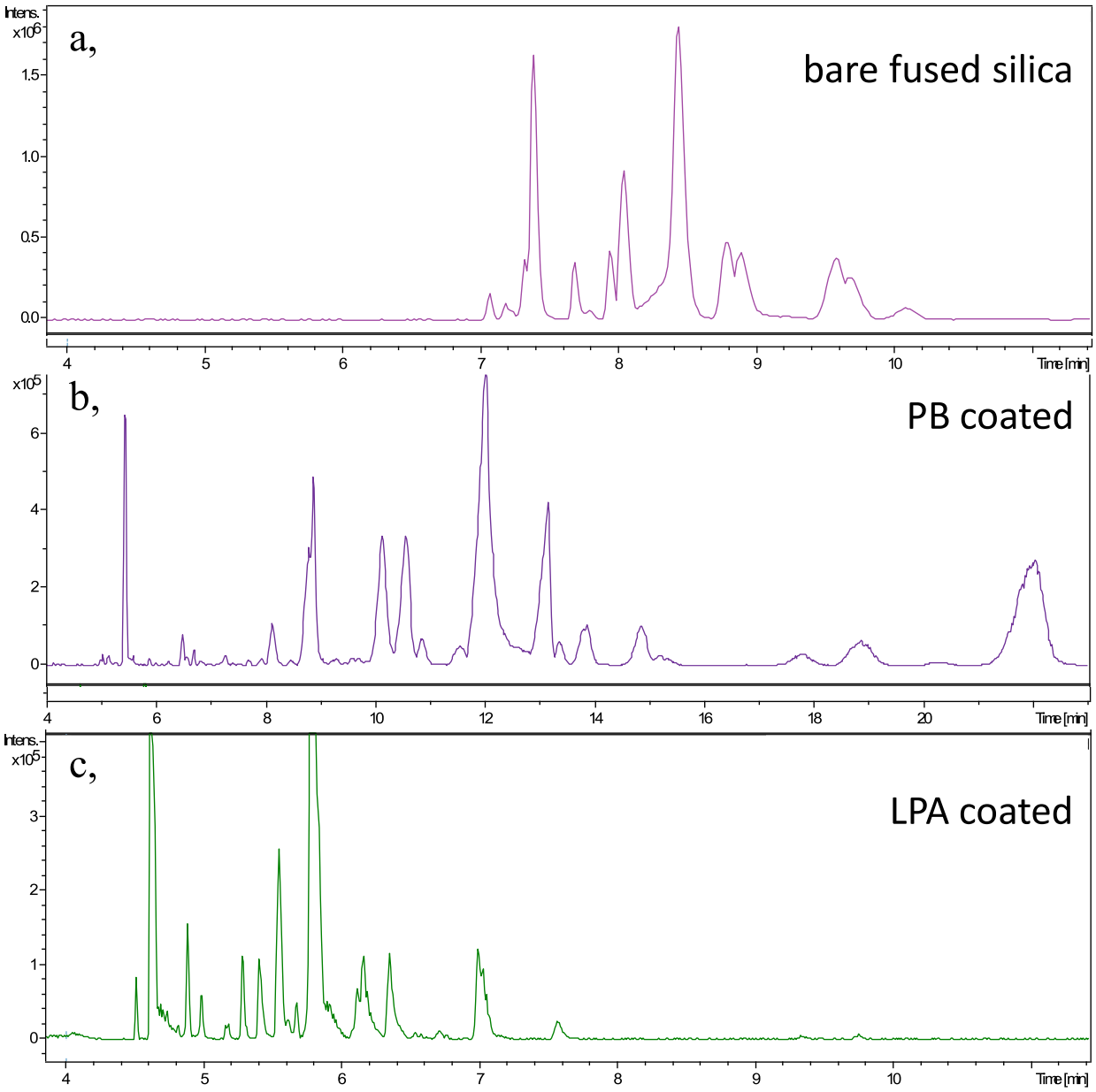

Fig. 6. The comparison of cobra venom analyses in different capillaries: **a:** bare fused silica, BGE: 1 M formic acid; **b:** polybrene coated, BGE: 1 M acetic acid; **c:** LPA coated capillaries, BGE: 0.05 M formic acid. Sample: cobra snake venom (#304, 10x diluted). The CZE conditions were same as in Fig. 4. and MS parameters were identical as in Fig. 3. The detailed mass list of the peaks is shown in Fig. SM-11.

of proteins or peak broadening could be observed (Fig. SM-6.h.-j.) The smaller shift of migration times can be effectively compensated by the use of internal time reference marker(s) or formation of a triple layer coating (SMIL) [33]. The SMIL technique improved the precisions of the migration times considerably and the peak areas slightly (Table SM-3, Fig. SM-7).

With LPA capillary (similarly as with PB modified capillary) an enhanced separation efficiency compared to those obtained with bare fused silica capillary can be represented by resolving the different hemoglobin subunits and variants (Fig. 5). In the case of hemoglobin variants the PB coating yielded better separation between the α_a and β_a' than LPA coating.

Based on the obtained efficiency data (Table 1) we concluded that the separation efficiencies in the different capillaries could be in a relatively similar range for a given protein (e.g., 7 000/m - 27 000/m for insulin), but those can be also very different for other proteins (e.g., 39 000/m - 322 000/m for myoglobin). The highest plate numbers were obtained in the bare fused silica capillaries. However, the best resolutions for the different forms of a protein (e.g., hemoglobin variants) were obtained in PB or LPA coated capillary. For understandig this result further investigations are needed.

For the optimization of the separation, the proper capillary length which ensures both good separation efficiency and connectivity of CE with MS (minimum 65 cm but typically 85-100 cm) should be considered. For UV detection the capillary length is minimum 30 cm or it can be 8 cm with short-end injection. Increasing the capillary length generally improves the resolution of the separation but prolongs the analysis time (Fig. SM-8.-10.). The best resolution between the several subunits of albumin, myoglobin and hemoglobin was obtained with 85 cm LPA capillary (Fig. SM-10.).

3.3. Comparison of the separations of proteins in venom sample using different capillaries

Snake venom is a natural product with an extremely high and diverse protein content [38]. Fig. 6 shows the CE-MS measurements of a cobra venom using all three studied capillaries. All three capillaries were suitable for the separation of several proteins and other components. The bare fused silica capillary and LPA coated capillary enabled the separation of 32 components (29 and 31 proteins with non-coated and LPA coated capillaries, respectively); and even more, 52 components (38 proteins) could be separated using PB coated capillaries (the detailed mass list of the peaks is shown in Fig. SM-11.). The larger peak capacity achieved with PB coating can be explained with the larger time window and/or the better resolving performance for smaller (not proteins/peptides) molecules.

While ESI-MS is relatively sensitive for peptides and smaller proteins (<25 kDa), poor detection sensitivities might be obtained for larger proteins that present a wide range of charge state distibution (to which adduct formation also contributes). This is the reason why although venom includes a lot of larger proteins (e.g., snake venom metalloproteinases (SVMPs) range from 20 to 100 kDa), those can not be detected with MS. With UV detection these large proteins are detectable but not identifiable (Fig. SM-12). (The number of the separated components were similar for UV and MS detection since, on the one hand, MS could sense the ionisable components only in the 600-2500 m/z range, excluding the large proteins; on the other hand, even though UV could detect even the large proteins, it obviously failed to detect smaller components lacking a chromophore group.)

4. Conclusion

In this study, we compared the applicability and analytical performance of bare fused silica, dynamically coated (PB) and permanently coated (LPA) capillaries for the separation of intact proteins. For these studies five proteins (with quite different sizes and isoelectric points) and for some experiments a very large protein – thyroglobulin - were used.

Although, in general, the utilization of coated capillaries is advised for the separation of proteins, the use of bare silica capillary is still dominant due to its simplicity and low-cost. In this work we demonstrated that when the separation of proteins is performed in bare fused silica capillary at a very low pH (pH=1.8), minimal adsorption of components, good precision and separation efficiency similar to or even better than those obtained with the coated capillaries (created by an intricate multi-step process) were achieved. The bare fused silica capillary provided good separation efficiency and precision only at this low pH, the use of BGE with pH>2.5 demonstrated stronger adsorption and poorer precision. Therefore there is no need to optimize the pH for the separation of proteins, only the 0.5-1 M FA could be recommended as BGE. This low pH BGE could be well applied for the separation of a large number of proteins. Although the short term precision obtained with bare fused silica capillary was good and similar to those of the coated capillaries, we found that the separation performance deteriorates if the capillary was not used for a few days (dried out). When a remarkable decrease of the separation efficiency (shifted migration times) is observed, the capillary should be replaced (regeneration of the capillary utilizing extended washing periods with concentrated NaOH, H_2O_2 or Piranha solution or etching with HF/NH_4HF_2 did not help). The disposal of a capillary even after a few-day use might be affordable, since a 100 m length of bare fused silica capillary is cheaper than a HPLC column or a commercially available coated CE capillary. It was demonstrated that the proper postconditioning of the capillary after the separations is important, especially for bare fused silica capillaries. Comparing different postconditioning procedures, flushing the bare silica capillary with strong base and/or strong acid is highly advised. Although it was showed that the BGE with low pH is favourable for the separation of proteins, peptides and small proteins might be efficiently separated at basic conditions (pH>9), as well (in our recent work even the deamidation forms of insulin having an identical molecular mass of 5.5 kDa and net charge could be separated in a bare fused silica capillary [39]).

We found that the most common coated capillaries (PB, LPA) provided relatively similar efficiencies and precision to those of bare silica capillaries. In several papers the superior performance of the proposed coatings were demonstrated based on the comparisons of separations carried out using the conditions optimal for the coated capillary only, disregarding the low pH demand of the bare silica capillary.

The PB and the LPA capillaries demonstrated their excellent separation power where high separation efficiency was needed, for instance, in the case of the separation of the different forms/variants of the same protein (e.g., hemoglobin subunits). Among the studied capillaries, the LPA coated capillary showed the most stable separations in the long term. But for the separation of a few proteins (5-protein mixture) or even a larger number of proteins in a biological sample (snake venom) the application of the simple and cheap bare fused silica capillary should not be considered as an inefficient choice.

Credit author statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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