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Modelling the electrophoretic migration behaviour of peptides and glycopeptides from glycoprotein digests in capillary electrophoresis-mass spectrometry

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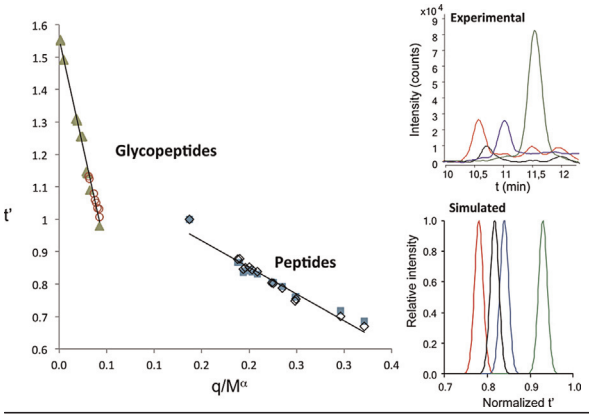
HIGHLIGHTS

- Semiempirical models are used to predict rhEPO peptide–glycopeptide migration times.
- The Stoke's law ($q/M^{1/3}$) result in better linear correlations for rhEPO peptides.
- rhEPO glycopeptides fit better with the classical polymer model ($q/M^{1/2}$).
- These models permit to simulate rhEPO peptide and glycopeptide CE separations.
- Potential applicability to estimate electrophoretic maps of other digested glycoproteins.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, the classical semiempirical relationships between the electrophoretic mobility and the charge-to-mass ratio (m_e vs. q/M^{α}) were used to model the migration behaviour of peptides and glycopeptides originated from the digestion of recombinant human erythropoietin (rhEPO), a biologically and therapeutically relevant glycoprotein. The Stoke's law ($\alpha = 1/3$), the classical polymer model ($\alpha = 1/2$) and the Offord's surface law ($\alpha = 2/3$) were evaluated to predict migration of peptides and glycopeptides, with and without sialic acids (SiA), in rhEPO digested with trypsin and trypsin–neuraminidase. The Stoke's law resulted in better correlations for the set of peptides used to evaluate the models, while glycopeptides fitted better with the classical polymer model. Once predicted migration times with both models, it was easy to simulate their separation electropherogram. Results were later validated predicting migration and simulating separation of a different set of rhEPO glycopeptides and also human transferrin (Tf) peptides and glycopeptides. The excellent agreement between the experimental and the simulated electropherograms with rhEPO and Tf digests confirmed the potential applicability of this simple strategy to predict, in general, the peptide–glycopeptide electrophoretic map of any digested glycoprotein.

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1. Introduction

Capillary electrophoresis mass spectrometry (CE–MS) is nowadays a mature technique for the analysis of peptides and proteins [1–8], that has been widely explored as an alternative to liquid chromatography mass spectrometry (LC–MS) for separation and characterization of protein digests in the typical bottom-up strategies applied in proteomic analysis [5–10]. The recent development of novel nanoESI sheathless interfaces is revitalising the interest in CE–MS as a high-separation efficiency and sensitive tool to achieve high sequence coverage of minute diluted protein digests [3,7,8,11]. Furthermore, several authors have demonstrated the excellent performance of CE–MS for the analysis of glycopeptides from glycoprotein digests [11–15].

Several authors have regarded prediction of electrophoretic migration behaviour of the compounds of interest, including peptides, as an excellent tool to speed-up method development in CE–MS, as well as to refine the structural assignments made on the basis of the measured molecular mass (M) [16–24]. Migration of ionisable compounds in capillary zone electrophoresis (CZE) can be easily predicted using the classical semiempirical relationships between the electrophoretic mobility and charge-to-mass ratio (m_e vs. q/M^{α}) or other models, such as those based on the Hubbard–Onsager dielectric friction theory of ion mobility [4-6,16–34]. In our previous works, the Stoke's law ($\alpha = 1/3$, peptides are modelled as spherical particles), the classical polymer model ($\alpha = 1/2$, peptides are polymers with lower q densities) and the Offord's surface law ($\alpha = 2/3$, for larger and more rigid structures, which experience frictional forces that are proportional to their surface area) yielded excellent correlations when they were employed to study the m_e of several peptide hormones [18], neuropeptides [31], quinolones [32] and metallothioneins [35], when good estimates of acidity constant values were available for charge calculations. Migration prediction of peptides from protein hydrolysates has been also explored by several authors [4-6,20–24], but those works were mainly focused in the analysis of proteins without post-translational modifications (PTMs) [20–23]. To the best of our knowledge, only J. Kim et al. evaluated before several semiempirical models with post-translationally modified peptides resulting from tryptic digestion of human myelin basic protein, taking into account citrullination, deamidation, oxidation, phosphorylation and methylation [24]. Modelling migration behaviour of glycoprotein digests, which are complex mixtures of peptides and glycopeptides [11–15], represents a further challenging task. On the one hand, we have the evident structural dissimilarities between peptides and glycopeptides. On the other hand, glycopeptides show up as a mixture of glycoforms due to the different composition of the carbohydrate chains attached to the peptide core [14,15,36–40]. Thus, while the number and type of glycans of the carbohydrate chains affects glycoform size, and hence M, the specific presence of sialic acid (SiA = *N*-acetylneuraminic acid) strongly contributes to their charge and it is critical for the electrophoretic separation resolution [14,15].

In this paper, we investigated the classical semiempirical relationships between m_e and q/M^{α} to model the migration behaviour in CE–MS of peptides and glycopeptides originated from the digestion of recombinant human erythropoietin (rhEPO), a biologically and therapeutically relevant glycoprotein [11–15]. The aim is to describe a simple strategy to easily generate 'dry-lab' peptide–glycopeptide electrophoretic maps of glycoproteins, which can be later useful to assist separation optimization and identification.

2. Materials and methods

2.1. Chemicals

All chemicals used in the preparation of buffers and solutions were of analytical reagent grade. Isopropanol (iPrOH, ≥99.9%), acetic acid (HAc, glacial), formic acid (HFor 98–100%), ammonia (25%), ammonium acetate (NH_4Ac, ≥99.99%) and sodium hydroxide (≥99%) were supplied by Merck (Darmstadt, Germany). DL-Dithiothreitol (DTT, ≥99%), iodoacetamide (IAA, ≥98%), ammonium hydrogen carbonate (≥99.9%) and human Tf standard (≥98%) were supplied by Sigma–Aldrich (Madrid, Spain). Trypsin (sequencing grade modified, 16,000 U mg^{-1}) from Promega (Madison, WI, USA) and neuraminidase (sialidase, 100 U mg^{-1}) were obtained from Roche (Mannheim, Germany). Water with a conductivity lower than 0.05 mS cm^{-1} was obtained using a Milli-Q water purification system from Millipore (Molsheim, France). ESI Low Concentration (ESI-L) tuning mix was supplied by Agilent Technologies (Waldbronn, Germany) for tuning and calibration of the TOF mass spectrometer.

2.2. Protein samples

rhEPO produced in a Chinese hamster ovary (CHO) cell line was provided by the European Pharmacopoeia as a Biological Reference Product (BRP-lot3). Each sample vial contained 250 μg of EPO (a mixture of epoetin alpha and beta), 0.1 mg of Tween 20, 30 mg of trehalose, 3 mg of arginine, 4.5 mg NaCl, and 3.5 mg of Na_2HPO_4. The content of each vial was dissolved in water to obtain a 1000 mg L^{-1} solution of rhEPO. Excipients of low molecular mass were removed from the rhEPO sample by passage through a Millipore Microcon YM-10 centrifugal filter (molecular weight cut-off, MWCO, 10 kDa) [14,15]. All the following centrifugation steps were performed for 10 min at 13,000 × g unless otherwise indicated. First, the filter was washed with water before loading the sample. After sample filtration, the sample residue was washed three times with an appropriate volume of water. The final residue was recovered from the upper reservoir by upside-down centrifugation in a new vial (3 min at 1000 × g). Finally, sufficient water was added to adjust the rhEPO concentration to 1000 mg L^{-1}.

rhEPO was reduced, alkylated and immediately subjected to enzymatic digestion [14,15]. Briefly, 2.5 μL of 0.5 M DTT in 50 mM NH_4HCO_3 (pH 7.9) were added to an aliquot of 100 μL of the filtered 1000 mg L^{-1} rhEPO solution. The mixture was incubated in a water bath at 56 °C for 30 min and then alkylated in 50 mM IAA for 30 min at room temperature in the dark (7 μL of 0.73 M IAA were added). Excess of low molecular mass reagents was removed with Microcon YM-10 centrifugal filters as explained before. Tryptic digestion (rhEPO-T digest) [14,15]: the final residue was reconstituted in 100 μL of 50 mM NH_4HCO_3 (pH 7.9). Trypsin was added in an enzyme to sample ratio of 1:40 w/w, the mixture was carefully vortexed and later incubated at 37 °C in a water bath for 18 h. Digestion was stopped by heating for 5 min in boiling water and stored at −20 °C until its use [14,15]. Neuraminidase digestion (rhEPO-TN digests) [14,15]: SiA residues were released from rhEPO tryptic glycopeptides by enzymatic digestion with neuraminidase [15]. Once rhEPO sample was subjected to tryptic digestion, sample was evaporated to dryness with air and reconstituted with 100 μL of 50 mM NH_4Ac (pH 5.0). Subsequently, 1 μL of neuraminidase (50 mU) was added, and solution was incubated at 37 °C for 18 h. Digestion was stopped by heating for 5 min in boiling water and stored at −20 °C until its use [15]. T- and TN-digests were obtained in different days.

Tryptic digests of human Tf (Tf-T) were obtained following the procedure described above for the tryptic digestion of rhEPO.