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Advanced portrayal of SMIL coating by allying CZE performance with in-capillary topographic and charge-related surface characterization

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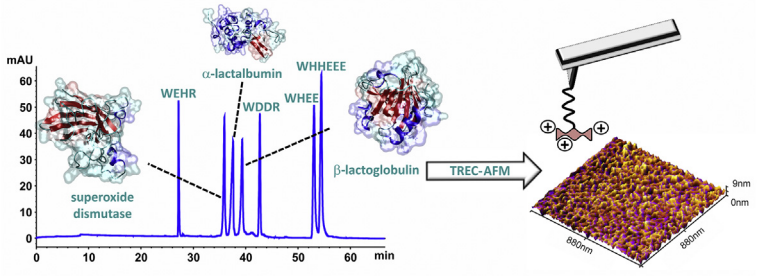
HIGHLIGHTS

- SMIL coating with a terminal layer of reduced charge density improves CZE separation.
- Capillaries with rectangular diameter allow for in-capillary TREC-AFM measurement.
- CZE performance is related to topography and charge distribution on the SMIL surface.
- Topographic changes are confirmed by statistical methods.

GRAPHICAL ABSTRACT



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ABSTRACT

A successive multiple ionic polymer layer (SMIL) coating composed of four layers improved the capillary electrophoretic separation of a recombinant major birch pollen allergen and closely related variants when poly(acrylamide-co-2-acrylamido-2-methyl-1-propansulfonate) (55% PAMAMPS) replaced dextran sulfate as terminal SMIL layer. 55% PAMAMPS decelerated the electroosmotic flow (EOF) due to its lower charge density. Atomic force microscopy (AFM) was used to investigate SMIL properties directly on the inner capillary surface and to relate them to EOF measurements and results of associated CZE separations of a mixture of model proteins and peptides that were performed in the same capillary. For the first time, AFM-based biosensing topography and recognition imaging mode (TREC) under liquid conditions was applied for a sequential characterization of the inner surface of a SMIL coated capillary after selected treatments including pristine SMIL, SMIL after contact with the model mixture, after alkaline rinsing, and the replenishment of the terminal polyelectrolyte layer. A cantilever with tip-tethered avidin was used to determine the charge homogeneity of the SMIL surface in the TREC mode. SMIL coated rectangular capillaries with 100 μm internal diameter assured accessibility of the inner surface for this cantilever type. Observed changes in CZE performance and EOF mobility during capillary treatment were also reflected by alterations in surface roughness and charge distribution of the SMIL coating. A renewal of the

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terminal SMIL layer restored the original surface properties of SMIL and the separation performance. The alliance of the novel TREC approach and CZE results allows for an improved understanding and a comprehensive insight in effects occurring on capillary coatings.

1. Introduction

CE and particularly CZE are progressively applied in the analysis of peptides [1,2] and intact proteins [3,4]. The miniaturized capillary diameter, efficient heat dissipation and the application of high electric fields in combination with the EOF allow for fast separations of high efficiency with only minute sample and electrolyte consumption. However, electrostatic adsorption of proteins onto the inner capillary surface impairs repeatability, efficiency, and electrophoretic resolution of CZE separations [5,6]. Different strategies have been applied to combat protein adhesion, including (i) convenient rinsing protocols before and after CZE runs to diminish adsorption and detach adhered proteins [6–8], application of background electrolytes (BGEs) of appropriate (ii) pH and (iii) ionic strength [9–11], but also passivation of the silica surface. In the latter case, (iv) dynamic coatings primarily using oligoamines [9,12,13], (v) physical adhesion of single polymer layers [14,15], and (vi) covalent coatings [16–18] have been employed. However, these strategies include individual drawbacks, i.e. the addition of dynamic coating reagents to the BGE and thus incompatibility with MS, restricted durability of adhered polymers with the concomitant need for frequent recoating, or laborious preparation of covalent coatings with possible performance variation between individual capillaries and the incapability of coating regeneration after its decay [19,20].

Katayama et al. [20,21] have developed an innovative successive multiple ionic polymer layer (SMIL) coating. Thereby, countercharged polyionic polymers are successively adhered onto the inner capillary surface thus generating coatings composed of alternating polycationic and polyanionic layers. Their Coulomb interaction with the respective underneath layer occurs by multipoint contacts of defined segments, so-called trains. Interjacent polymer segments form loops that protrude towards the bulk solution together with the terminal loose tail [22]. The conformation of adhered polyelectrolytes (PEs), and thus the extent of loop protrusion, layer thickness and surface charge are targeted by the type of the PE and the ionic strength of the deposition electrolyte [23–25]. The influence of the pH as well as of the ionic strength of the surrounding bulk solution on the layer thickness and the amount of adsorbed PE has been tested by cycling the respective parameters [26]. The PE loops ensure the entanglement of the PE layers and thus a firmly interwoven SMIL architecture that improves the coating durability as well as its resistance to rinsing solutions in comparison to monolayers [20]. The selected PEs and the coating procedure substantially influence the SMIL performance and durability [27,28]. SMIL coatings have successfully been applied in the separation of peptides [28,29] and (recombinant) proteins [21,25,27] proving also MS applicability but required frequent recoating, e.g. by overnight storage in a solution of the PE that constitutes the terminal SMIL layer [30]. A thoroughly optimized SMIL coating protocol of our group included *inter alia* resting steps for adhered SMIL layers and voltage application between the consecutive attachments of individual PE layers. As a result, a four-layer SMIL coating with a durability of several weeks was generated without the need for recoating in between [27]. Generally, SMIL coated capillaries possess a strong μ_{EOF} that is less pH dependent than for bare fused-silica capillaries when using strong PEs of high molecular weight as terminal layer and appropriate ionic strength during the deposition [23]. Although SMIL coatings enhance the separation repeatability they deprive the operator of μ_{EOF} tuning for improving electrophoretic resolution particularly for closely related analytes, e.g. PTM variants. The group of H. Cottet has synthesized and tested statistically charged copolymers of reduced charge densities to overcome this limitation [31]. However, the balance between satisfactory SMIL stability and adequate μ_{EOF} retardation is delicate and the charge density of the PE has to exceed a critical value to achieve a repeatable EOF. So far, SMIL coatings with a terminal layer of reduced charge density have been tested with synthetic peptides [31] but not with intact proteins.

Generally, the evaluation of the capillary coating should not only address the separation performance but additionally its surface properties. In this context, atomic force microscopy (AFM) is an appropriate technique [32]. Currently, bare fused-silica and coated silica surfaces have only been characterized on basis of their topography [33] and the related homogeneity via the root mean square (RMS) that is linked to the average surface roughness [32]. Topographic imaging has primarily been done for coated glass slides [34,35], silica wafers [33,36,37], and the outer surface of fused-silica capillaries [31,38]. However, the effect of capillary rinsing protocols and particularly of voltage application in combination with the short-term contact between concentrated protein and peptide plugs and the inner capillary surface as typically occurring during CZE separations cannot be mimicked this way. To overcome this limitation, some authors smashed capillaries after removal of polyimide with hot sulfuric acid or fragmented the capillary prior to AFM measurements. By this preparation surface artifacts cannot be excluded [39,40]. Others gained access to the capillary lumen by cleaving or splitting the capillary axially with an appropriate tool thereby receiving longitudinally intact fractions or by stepwise grinding of an epoxide embedded capillary. This way bare and coated inner capillary surfaces but also their topography after protein adsorption has been investigated [41–43]. Surface characterization by AFM has been performed both in dry state [31,38,40,44,45] and in fluid [33,36,38]. Up to now, all AFM-based evaluations were only confined to the surface roughness of the capillary coating but excluded consequences on the surface net charge. This is related to the fact that primarily neutral coatings, e.g. polyacrylamide, have been applied in combating protein adhesion onto bare fused-silica surfaces in CE [33]. Additionally, AFM has been used to characterize changes in the topography and roughness of the inner capillary surface during individual reaction steps in covalent coating [42] to analyze the influence of selected coating parameters [45], and to evaluate the final homogeneity of the coating topography [40], all for neutral polymers. In case of ionic polymer layers and particularly for SMIL coatings [20,21] the surface charge constitutes a crucial aspect in relating observed CZE separation results to changes in the coating architecture.

This work aims to investigate the applicability of SMIL coating with a terminal polyanionic PE layer of reduced charge density in the separation of closely related proteins, e.g. variants of the major birch (*Betula verrucosa*) pollen allergen Bet v 1a, and selected model proteins and peptides of related isoelectric points, i.e., 4.05–7.00.