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Surface charge fine tuning of reversed-phase/weak anion-exchange type mixed-mode stationary phases for milder elution conditions

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ABSTRACT

A series of new mixed-mode reversed-phase/weak anion-exchange (RP/WAX) phases have been synthesized by immobilization of *N*-undecenyl-3-α-aminotropane onto thiol-modified silica gel by thiol-ene click chemistry and subsequent introduction of acidic thiol-endcapping functionalities of different type and surface densities. Click chemistry allowed to adjust a controlled surface concentration of the RP/WAX ligand in such a way that a sufficient quantity of residual thiols remained unmodified which have been capped by thiol click with either 3-butenoic acid or allylsulfonic acid as co-ligands. In another embodiment, performic acid oxidation of *N*-undecenyl-3-α-aminotropane-derivatized thiol-modified silica gave a RP/WAX phase with high density of sulfonic acid end-capping groups. ζ-Potential determinations confirmed the fine-tuned pI of these mixed-mode stationary phases which was shifted from 9.5 to 8.2, 7.8, and 6.5 with 3-butenoic acid and allylsulfonic acid end-capping as well as performic acid oxidation. For acidic solutes, the co-ionic endcapping leads to strongly reduced retention times and clearly allowed elution of these analytes under lower ionic strength thus milder elution conditions. In spite of the acidic endcapping, the new mixed-mode phases maintained their hydrophobic and anion-exchange selectivity as well as their multimodal nature featuring RP and HILIC elution domains at acetonitrile percentages below and above 50%, respectively. Column classification by principal component analysis of an extended retention map in comparison to a set of polar commercial and in-house synthesized stationary phases confirmed complementarity of the new mixed-mode phases with respect to HILIC, polar RP, amino and commercial mixed-mode phases.

1. Introduction

Modern analytical liquid HPLC and UHPLC of organic molecules is strongly dominated by reversed-phase liquid chromatography (RPLC). Efficient separation of organic solutes such as drugs, peptides and even proteins based on lipophilicity differences with excellent (methylene and/or hydrophobic) selectivity and good mass spectrometric detection compatibility make this LC mode the first choice in the field. Many of past research attempts focused on the endeavor of eliminating retention contributions other than originating from hydrophobic interactions between alkyl strands of the adsorbent and lipophilic moieties in the analytes (i.e. primarily H-bond and cation interactions with residual silanols). While this has led to highly efficient columns providing narrow peaks with minimal tailing for average compounds, retention and selectivity profiles of C18 phases from one supplier compared to the other became very similar, virtually interchangeable [1,2]. This is connected with severe limitations if a complementary stationary phase is required for impurity profiling or multidimensional separation applications, which rely on orthogonality in retention and selectivity profiles [3–9]. For hydrophilic compounds, HILIC is nowadays commonly adopted as the prime LC separation mode when RPLC fails [10–13]. It is based on a distribution mechanism of solutes between acetonitrile-rich aqueous mobile phase and an adsorbed water layer on the polar stationary phase surface. However, additional specific non-covalent analyte–stationary phase interactions lead typically to a kind of mixed partitioning-adsorption mechanism [10,14,15]. This mixed interaction mechanism may provide some complementarity in retention profiles of different HILIC phases, because surface chemistries of HILIC-type adsorbents vary greatly [10]. Still, if the partitioning mechanism is dominating, orthogonality in retention profiles is modest [16,17].

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In order to enable a better balancing and controlling as well as a more systematical exploitation of such mixed interaction mechanisms, researchers have introduced mixed-mode chromatography, which utilizes stationary phases that combine two distinct retention principles such as reversed-phase and ion-exchange [18–30] or HILIC and ion-exchange [31,32]. Also stationary phases with immobilized ionic-liquids can be placed into this category of mixed-mode ion-exchangers [33,34]. Note that mixed-mode ion-exchangers exhibit strong retention for counterionic analytes for which particular selectivities can be achieved as compared to RP and for the separation of which they have actually been primarily designed. It requires high buffer concentration in the eluent to balance strong electrostatic interactions. Unfortunately, such conditions are not compatible for MS hyphenation which prefers low ionic strength conditions ($\leq$10 mM) for stable electrospray and highly abundant MS signal intensities, respectively [35,36]. The problem is deemed to be overcome in this study by introduction of co-ionic co-ligands, which balance strong, attractive electrostatic counterionic solute–sorbent interactions through (i) long-range (electrostatic) counterion effects, and/or (ii) a negative retention increment originating from repulsive electrostatic interactions at co-ligand site, which is additively superimposed upon the adsorption increment at the counterionic anion-exchanger site.

To some extent, this principle has been implemented in concepts such as charged surface hybrid (CSH) stationary phases [37], in which the C18 phase is doped with positively charged groups, or electrostatic repulsion hydrophilic interaction chromatography (ERLIC) [38]. A theoretical discussion and modeling of adsorption for charged RP phases (fixed charges on RPC18) has been reported recently by Gritti and Guiochon for small molecules [39] and large molecules [40]. In protein chromatography, repulsive electrostatic interactions are exploited during the elution step in the chromatographic mode known as hydrophobic charge induction chromatography (HCIC) [41]. Furthermore, there are reports on peptide separations by mixed-mode chromatography in which electrostatic repulsion has been favorably utilized as component of the entire mixed-mode adsorption and retention process [23,42]. In other attempts, dissociation of residual surface silanols has been induced in the course of pH gradient elution (from low pH to high pH) on silica-based weak anion-exchangers in order to activate repulsive electrostatic interactions facilitating analyte desorption by repulsive charge-inducted elution of highly negatively charged oligonucleotides and plasmid DNA on reversed-phase/anion-exchange type mixed-mode phases and anion-exchange based affinity-type separation media, respectively [24,43]. This allowed more efficient elution of biomolecules from counterionic chromatographic surfaces with significantly enhanced recovery.

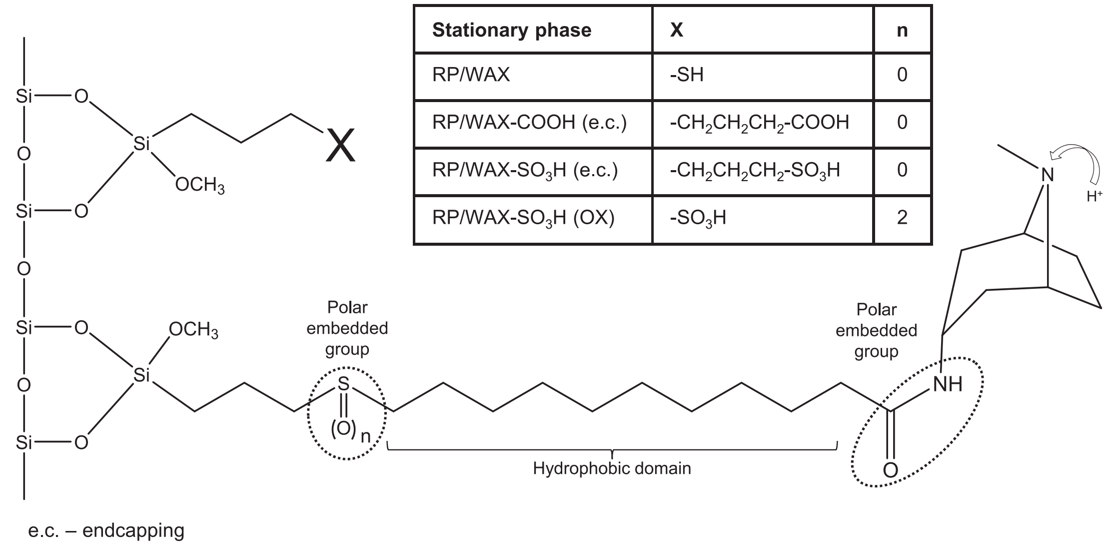
In the present work, new stationary phases for mixed-mode chromatography are proposed. They are based on an RP/WAX ligand derived from *N*-undecenoyl-3-α-aminotropane immobilized on 3-mercaptopropyl-modified silica by thiol-ene click chemistry (Fig. 1). The net surface charge of the mixed-mode phase has been balanced by the introduction of ionic co-ligands with opposite charge to the one of the mixed-mode RP/WAX ligand as end-capping groups (Fig. 1). The resultant zwitterionic surfaces [44–50] revealed carefully shifted pI values in dependence on the type and density of the introduced ionic endcapping groups, respectively. The new mixed-mode phases have been characterized by elemental analysis, pH-dependent ζ-potential determinations and by liquid chromatographic studies at distinct organic modifier, pH and counterion concentrations. Retention profiles of a set of test compounds were analyzed by principal component analysis (PCA) in comparison to a number of common HILIC, RP and mixed-mode phases. PCA allowed the classification of the new phases in relation to those commonly employed in RPLC and HILIC.

2. Materials and methods

2.1. Materials

3-Mercaptopropyltrimethoxysilane was supplied by ABCR (Karlsruhe, Germany). 3-α-Aminotropane dihydrochloride was from Boehringer-Ingelheim (Ingelheim, Germany). Sodium allylsulfonate was purchased from TCI Chemicals (Eschborn, Germany). 2,2′-Azobis(2-methylpropionitrile) (AIBN), 3-butenoic acid, 2,2′-azobis(2-methylpropionamidine)dihydrochloride (AAPH), tris-(2-carboxyethyl)phosphine (TCEP), sodium dihydrogen phosphate, formic acid and hydrogen peroxide solution were supplied by Sigma Aldrich (Munich, Germany).

The test compounds (butylbenzene, pentylbenzene, adenosine, guanosine, cytidine, thymidine, uridine, ascorbic acid, nicotinic acid, pyridoxine, riboflavin, thiamine, caffeine, theobromine, theophylline, L-phenylalanine (Phe), *N*-acetyl-L-phenylalanine (AcPhe), L-phenylalanine methyl ester (PheOMe), L-tryptophan (Trp), *N*-acetyl-L-tryptophan (AcTrp), L-tryptophan-amide



Stationary phase	X	n
RP/WAX	-SH	0
RP/WAX-COOH (e.c.)	-CH$_2$CH$_2$CH$_2$-COOH	0
RP/WAX-SO$_3$H (e.c.)	-CH$_2$CH$_2$CH$_2$-SO$_3$H	0
RP/WAX-SO$_3$H (OX)	-SO$_3$H	2

Fig. 1. Chemistries of mixed-mode RP/WAX phases.