Polyhydroxyl and Polyphosphorylcholine Functionalized Silica for Hydrophilic Interaction Liquid Chromatography

Synthesis, Characterization and Application

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Abstract

This thesis focuses on the development of new stationary phases for use in hydrophilic interaction liquid chromatography using TRIS-based and phosphorylcholine typed monomers and porous silica particles as starting substrates. In this thesis, several ways of polymerizing highly hydrophilic monomers onto pore surfaces of silica supports are described, based on several “grafting from” schemes. “Controlled/living” radical polymerizations including atom transfer radical polymerization (ATRP) and iniferter-mediated polymerization in conjunction with conventional free radical polymerization are demonstrated to be successful tools for grafting different hydrophilic monomers (polyhydroxyl and phosphorylcholine [meth]acrylamide/acrylates) onto the silica surfaces. Reaction solvents are proven to play an essential role to achieve efficient graft polymerization of activated silica surfaces with these amphiphilic vinylic monomers, which is difficult because of their restricted access to the activated surface in solvents that can be used because of solubility constraints.

Two tentacle TRIS-based polymer grafted silica, namely TRIS-WAX – TRIS functionality bonded to silica via a C–N–C imine bond and TRIS-Amide – TRIS bonded to silica via an amide bond, prove to be useful as stationary phases for hydrophilic interaction chromatography (HILIC). The TRIS-WAX exhibits a mixed mode hydrophilic partitioning and weak anion exchange (HILIC/WAX) retention mechanism while retention by hydrophilic partitioning is the dominant mechanism on the neutral TRIS-Amide phase which lacks weak anion exchange (WAX) properties. Interestingly, both these phases have selectivities that are radically different from most commercial HILIC stationary phases.

Finally, a method is demonstrated for synthesizing a stratified (graft-copolymerized) silica material based on N,N'-methylenebisacrylamide and 2-methacryloyloxyethyl phosphorylcholine (MPC) using a "controlled/living" photoiniferter-mediated polymerization from the N,N-diethylidithiocarbamate iniferter moiety immobilized silica surfaces. This polymerization method proves to be successful for graft-blockcopolymerization of different highly hydrophilic monomers onto the activated surfaces of porous silica. In this way, silica surfaces are grafted with a cross-linked amide-based hydrogel, on top of which a tentacle zwitterionic phosphorylcholine-type layer is synthesized. The resulted material proves to be useful for HILIC separations and possesses different selectivity for the tested organic acids compared to that of commercial ZIC-chILIC stationary phase.

Keywords: HILIC, silica, TRIS, acrylamide/acrylate, ATRP, iniferter, “controlled/ living” radical polymerization, N,N'-methylenebisacrylamide, MPC, stationary phase.

To Dads, Moms,
To My Dear Jeroen,
And My Sisters
List of Papers

This doctoral thesis is based on the following papers and manuscripts, which are hereafter referred to in the text by their Roman numerals.

I. **Tris(hydroxymethyl)aminomethane Functionalized Silica Particles and their Application for Hydrophilic Interaction Chromatography**
   Nhat Thi Hong Bui, Jeroen J. Verhage and Knut Irgum

II. **Synthesis of Poly(N-[Tris(hydroxymethyl)methyl]acrylamide) Functionalized Porous Silica for Application in Hydrophilic Interaction Chromatography**
    Nhat Thi Hong Bui, Wen Jiang, Tobias Sparrman and Knut Irgum
    *Journal of Separation Science, Accepted, 2012.*

III. **Retention and Selectivity of Polymeric Functionalized Silica Phases for Hydrophilic Interaction Chromatography**
    Nhat Thi Hong Bui, Hanh Thao Ho, Wen Jiang and Knut Irgum
    Manuscript

IV. **Synthesis of Graft-copolymerized Phosphocholine Type Zwitterionic Silica from N,N-Diethyldithiocarbamate group Immobilized Porous Silica by Photoiniferter Mediated Polymerization for Application in Hydrophilic Interaction Chromatography**
    Nhat Thi Hong Bui, Wen Jiang and Knut Irgum
    Manuscript

Paper I is reprinted with permisson from WILEY-VCH, Weinheim.
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine-5’-diphosphate</td>
</tr>
<tr>
<td>AIBN</td>
<td>α,α’-Azobis(isobutyronitrile)</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine-5’-monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated total reflectance</td>
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<tr>
<td>ATRP</td>
<td>Atom transfer radical polymerization</td>
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<tr>
<td>BE</td>
<td>Binding energy</td>
</tr>
<tr>
<td>BEH</td>
<td>Bridged ethylene hybrid</td>
</tr>
<tr>
<td>BET</td>
<td>Brunauer, Emmett, and Teller</td>
</tr>
<tr>
<td>BIS</td>
<td>N,N'-Methylenebisacrylamide</td>
</tr>
<tr>
<td>BJH</td>
<td>Barrett, Joyner, and Halenda</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cyclodextrin</td>
</tr>
<tr>
<td>CEC</td>
<td>Capillary electrochromatography</td>
</tr>
<tr>
<td>CLSP</td>
<td>ε-Amino tethered lysine phase</td>
</tr>
<tr>
<td>CMCH</td>
<td>Carboxymethyl chitosan</td>
</tr>
<tr>
<td>CRP</td>
<td>Controlled radical polymerizations</td>
</tr>
<tr>
<td>DC</td>
<td>Dithiocarbamate</td>
</tr>
<tr>
<td>DEDT</td>
<td>Diethyldithiocarbamate</td>
</tr>
<tr>
<td>DOPA</td>
<td>(2S)-2-amino-3-(3,4-dihydroxyphenyl)propanoic acid</td>
</tr>
<tr>
<td>DRIFT</td>
<td>Diffuse reflectance infrared Fourier transform</td>
</tr>
<tr>
<td>EDMA</td>
<td>Ethylene dimethacrylate</td>
</tr>
<tr>
<td>ELS</td>
<td>Electrophoretic light scattering</td>
</tr>
<tr>
<td>ESCA</td>
<td>Electron spectroscopy for chemical analysis</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FRP</td>
<td>Free radical polymerization</td>
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<tr>
<td>FT</td>
<td>Fourier transform</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GMA</td>
<td>Glycidyl methacrylate (2,3-epoxypropyl methacrylate)</td>
</tr>
<tr>
<td>HETP</td>
<td>Height equivalent to a theoretical plate</td>
</tr>
<tr>
<td>HILIC</td>
<td>Hydrophilic interaction (liquid) chromatography</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>IEC</td>
<td>Ion-exchange chromatography</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
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<tr>
<td>LDE</td>
<td>Laser Doppler electrophoresis</td>
</tr>
<tr>
<td>LDV</td>
<td>Laser Doppler velocimetry</td>
</tr>
<tr>
<td>γ-MAPS</td>
<td>3-Methacryloxypropyl trimethoxysilane</td>
</tr>
<tr>
<td>MPC</td>
<td>2-Methacryloyloxyethyl phosphorylcholine</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MSA</td>
<td>[2-(Methacryloyloxy)-ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NMP</td>
<td>Nitroxide-mediated polymerization</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance (spectroscopy)</td>
</tr>
<tr>
<td>NPC</td>
<td>Normal phase chromatography</td>
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<tr>
<td>ODS</td>
<td>Octadecyl silica</td>
</tr>
<tr>
<td>PALC</td>
<td>Per aqueous liquid chromatography</td>
</tr>
<tr>
<td>PEEK</td>
<td>Poly(etheretherketone)</td>
</tr>
<tr>
<td>PMDTA</td>
<td>N,N,N',N',N''-Pentamethyldiethylenetriamine</td>
</tr>
<tr>
<td>RAFT</td>
<td>Reversible addition-fragmentation chain transfer polymerization</td>
</tr>
<tr>
<td>RP</td>
<td>Reversed phase</td>
</tr>
<tr>
<td>RPLC</td>
<td>Reversed phase liquid chromatography</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SPE</td>
<td>N,N-Dimethyl-N-methacryloyethyl-N-(3-sulfopropyl)ammonium betaine</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra high pressure liquid chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WAX</td>
<td>Weak anion exchange</td>
</tr>
<tr>
<td>WCX</td>
<td>Weak cation exchange</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
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1. Introduction

Chromatography is a separation principle based on the difference in distribution of solutes transported by a mobile fluidic medium and a stationary phase. Solutes that are strongly distributed towards the stationary phase are consequently more retained than compounds spending the major part of the time in the mobile phase. In liquid chromatography, which this thesis deals with, the stationary phase is a material that can be percolated by the mobile phase, which is pumped through a bed of stationary phase by means of hydraulic force. Traditionally the stationary phase has been used in the form of a tubular column packed with a porous solid that is capable of withstanding the pressure drop, where the chemistry of the pore surfaces (or of an immobilized liquid trapped in the pore space) performing a task of attracting a mixture of compounds that have been dissolved in a liquid solution, usually a liquid mobile phase (eluent) onto its surface, whereby the compounds are retained on the stationary phase. The driving forces behind this retention are physical and/or chemical interactions between the solutes and the column. Separation takes place when the individual solutes have different affinity to the stationary phase (adsorption) or between the stationary phase and the mobile phase (partitioning).

High Performance Liquid Chromatography (HPLC) is a way of practicing liquid chromatography that utilizes small column sizes, material of smaller dimensions inside the column, and higher mobile phase pressures compared to open column liquid chromatography. The selection of mobile phases and stationary phases in HPLC is of importance since variations in these parameters are used to exploit different types of solute interactions. These interactions underlie all separations and give rise to the different separation modes in HPLC. The simplest interaction involves separation of solute molecules based on size and shape differences. This is called size exclusion chromatography (SEC), by some also termed gel filtration chromatography or gel permeation chromatography, depending on whether the eluent is aqueous or an organic solvent. Another means of separating molecules is based on their charge, an approach referred to as ion-exchange chromatography (IEC). Separations based on adsorptive interaction of the components with a polar and impervious stationary phase surface like alumina or silica gel, using relatively apolar solvents without intentional addition of water to the eluent, is called (non-aqueous) “normal phase” chromatography (NPC). For pervious stationary phases
where the interactive layer is either an immobilized liquid, or a swollen, liquid-like layer, solute-stationary phase interactions based on the dissolving power of one phase over the other causes solutes to partition or equilibrate themselves between the liquid mobile and (quasi)-liquid stationary phases. This is defined as partition chromatography, where separation is dependent on molecular ‘polarity’. When this partitioning is carried out between a non-polar stationary phase and an aqueous organic eluent, and retention arises from a combination of hydrophobic and solvophobic interactions with the two phases, it is referred to as “reversed phase” liquid chromatography (RP-HPLC). If a partitioning separation using a partly aqueous organic eluent is instead taking place with a hydrophilic ("polar") stationary phase, the separation method, which can be traced back to 1975 was originally considered a variant of NPC, and was later given the name “hydrophilic interaction chromatography” (HILIC) by Alpert in 1990. Two decades after the acronym was dubbed, separations in HILIC mode continue to gain interest and now sports a large number of applications due to its key advantages over other HPLC techniques. The solute retention mechanism in HILIC mode is now proven to be not only hydrophilic partitioning; other “polar” interactions including hydrogen bonding, dipole-dipole interaction, and electrostatic interactions are also involved in the retention mechanism. In order to facilitate these polar interactions, stationary phases used in HILIC make use of retained water layer, which has been deposited from the mobile phase, which typically has a solvent composition of about 5-40 % water in acetonitrile. The water trapped from the eluent is engaged on polar interactions either with polar surface functionalities of an impervious solid support such as silica, or take advantage of some hydrogen donor/acceptor and/or coulombic interactions with a layer that has been deposited on the solid support to enable for polar analytes to diffuse into the stationary phase layer where they are retained.

In order to selectively attract water from the eluent, the stationary phases in HILIC are therefore polar. The question is then, “How many stationary phases are used in HILIC?” With a vague answer of “Many” without an exact number, I would hope to refer the readers to a few recent prominent reviews on this topic in order to find detailed information and to assess the rapidly growing scope of HILIC as a separation mode in HPLC. Further down in this thesis, an effort will be made to give a summary on a few very recent stationary phases dedicatedly synthesized for HILIC. The reason for the abundant number of stationary phases dedicatedly synthesized for HILIC is that separation in HPLC is driven by


selectivity; each new stationary phase will potentially give new selectivity and hence variation in the phase composition that can be used to tune the retention patterns of polar solutes of widely different nature. This explains the aim of my thesis, namely to develop new stationary phases to widen the available selectivity range in HILIC. On the other hand, as mentioned above, the retention mechanisms in HILIC are complex and still under debate, mainly because of the lack of standardized HILIC phases that can be used for characterization and selectivity tests. The preparation and evaluation of new HILIC-type chromatographic materials is hence a step forward to widen our understanding about the retention mechanisms in HILIC and thus helps to improve the identification and classification of HILIC stationary phases, the prediction of the retention behavior on polar packings, and to extract the most influential variables to adjust optimal chromatographic conditions towards new chromatographic expert systems based on adaptive methods.

In this thesis, I have developed techniques for efficient graft polymerization of activated silica surfaces with hydrophilic and amphiphilic vinyl monomers for Hydrophilic Interaction Chromatography (HILIC). The synthesized materials have been characterized by various bulk and surface characterization techniques, including FT-IR spectroscopy, elemental analysis, nitrogen cryosorption, X-ray Photoelectron Spectroscopy (XPS) and Electrophoretic Light Scattering (ELS) for zeta potential measurements. When the polymer-grafted silica particles have been packed into HPLC columns and had their HILIC separation properties assessed, they have demonstrated an ability to separate a variety of polar compounds of different functionalities, including neutral nucleic bases and amino acids, strongly basic neurotransmitters, negatively charged nucleotides, and organic benzoic acids, providing high retention capacity and separation efficiency. Interestingly, each the synthesized polymer-grafted silica possessed a selectivity that was substantially different from the other and from all tested commercial HILIC phases.

2. High Performance Liquid Chromatography (HPLC)

2.1. History

The technique of chromatography is about 110 years old. The first reported experiments were reported in 1903, when the Russian botanist M. S. Tswett was
able to show separation of chlorophyll pigments on a column packed with chalk. The term chromatography is derived from the Greek noun 'chroma', which means 'color' and the verb 'graphein' which means 'to write' and is used to identify all laboratory techniques where distributions between stationary and mobile phases is used to separate mixtures of compounds. Revolution in the chromatography field by the Nobel Prize winning work of Martin and Synge in 1941 established a firm theoretical basis for the separation mechanism, and showed many practical improvements and hence increased speed and resolution. Gas chromatography (GC) was the first chromatographic technique developed from these improvements. As usage increased, improvements in both instrumentation and theories developed rapidly, and it eventually became apparent that GC has fundamental limitations by being applicable only to solutes that (in their native state or after proper derivatization reactions) are sufficiently volatile and thermally stable to be transported through the column in the gas phase. This could be avoided by using a liquid rather than a gas mobile phase, an insight that marked the birth of HPLC.

2.2. Advantages

HPLC has several advantages of over conventional low pressure liquid chromatography: The separation speed is faster due to improvements in stationary phase technology that has increased column efficiency so that shorter columns can be used in combination with higher mobile phase pressure. The resolution is largely maintained with these shorter columns by exploiting the inherent selectivity of different interaction modes. The smaller column dimensions offer high sensitivity by coupling with standard detectors such as UV and fluorescence and also with mass spectrometry, which allows detection of minute component concentrations.

2.3. Components of a liquid chromatograph

The basic parts comprising a liquid chromatograph can be divided into five main components: The solvent pump, the injector, the separation column, the detector, and data acquisition systems; see Figure 1 for a schematic representation of a basic HPLC system.

The column can be made either from a metal such as stainless steel, from a rugged polymer that is not swelled by the mobile phase, or from glass. One of the more important properties is that the internal walls in contact with the stationary phases (packing material) should be smooth to allow formation of an evenly and densely packed column bed during the packing procedure. Stationary phases are
available in many varieties of packing styles as well as chemical structures, and can be functionalized for added specificity.\textsuperscript{15}

Figure 1. Typical components of an HPLC system: 1, Column; 2, Sample injector; 3, Mobile phase pumping system; 4, Detector; 5, Electronic data recorder. Adapted from ref 14.

The four most commonly encountered packing material confections are shown in Figure 2, including solid particles, superficially porous (pellicular) particles, fully porous particles, and monoliths. The first type is a highly rigid material with an impermeable outer structure. The solid core and lack of diffusion limitation has a potential of providing ultra-high speed separations. However, due to the small surface area compared to porous materials, solid spheres can only offer very low the retention and limited sample loadability. The larger surface area found in superficially porous particles offers increased retention and better sample loading capacity, with a limited penalty in analysis time. Porous materials offer the utmost in retention and sample loadability, but the relatively long internal diffusion paths may lead to lower separation efficiency. Monolithic style columns are porous rod structures characterized by a bicontinuous network of support material transsected by macropores. The surface area needed to establish retention is contained as mesopores inside the material structure. Monolithic material offers high speed separation with low back pressure, but somewhat increased retention and sample loadability compared to columns packed with fully porous particles. Both particulate and monolithic material packings exist on the basis of silica and other inorganic carriers (alumina, zirconia, and titania) and based on organic polymers. One caution that should be noted with the rigid silica gel material is that it starts to dissolve already at neutral pH, and this dissolution is accelerated under basic conditions pH > 7.5). Separations can therefore only be performed below this pH.
Besides the material packing structure, another important feature affecting the separation is the particle size. Smaller particle size improves column efficiency and sensitivity\textsuperscript{16}.

![Figure 2. Four different structures of material packings in HPLC.]

Currently, the state-of-art particle diameter is about 1.7 µm, implemented with an ultra high pressure liquid chromatography (UPLC) system in order to withstand the much greater back pressures of around 100 MPa, compared to 20-30 MPa for traditional HPLC systems.

**Sample injectors.** In order to separate a mixture of components, a suitably sized sample containing the solutes needs to be transferred onto the top of the column. This requires an injector, which is positioned between the high pressure solvent pump and the column. Three different approaches to injection are stop-flow injection, septum injection, and loop injection. In the stop-flow injection, pumping of the eluent is halted, the top fitting of the column removed and the sample placed directly onto the stationary phase. Septum injection avoids the removal of the top fitting and is done while the mobile phase remains flowing by inserting a syringe needle through this septum and injecting its contents directly onto the column. Septum injections are furthermore restricted to relatively low operation pressures. Loop injection requires a multiple port switching valve and gives high reproducibility. It is therefore the preferred injecting method in HPLC.

**Mobile phase pumping systems.** The eluent flow is accomplished with a high pressure pump in order to overcome the resistance to fluid flow arising from the microparticulate stationary phase packed in a narrow bore column. Two major design variations found in all HPLC pumps are continuous displacement (syringe) pumps and intermittent displacement pumps.

**HPLC Detectors.** The task of the HPLC detector is to measure some property of the analyzed solutes that differs from that of the eluent, and to convert this into an electric output signal which is proportional to (or at least in predictable relation
to) the solute concentration in the column effluent. There are different approaches to HPLC detection. The most widely used techniques measure either refractive index changes or spectrophotometric absorption. Less common, but still commercially available, are fluorescence, electrical conductivity, and electrochemical detectors. A modern HPLC coupled with a mass spectrometer system gives best sensitivity, in addition to a vast amount of information that can be used to identify the analyzed compounds.

**Electronic data processing.** Once the detector signal is available in electronic form, it can be quantified, stored, and processed using dedicated data acquisition hardware and accompanying software. This increases data analysis accuracy and precision, while reducing operator attention. The software also facilitates quantification and calculation of performance parameters pertaining to the separation process.

### 2.4. Principles of a chromatographic separation

**Solute retention.** In a chromatographic process, the separation of a mixture is dependent on differences in retention of its components. Retention, which arises from selective interactions of each component with the stationary phase, is also affected by simultaneous migration of the flowing mobile phase. The retention is therefore a function of the fraction of time spent by a solute in the stationary and in the mobile phases. For a given HPLC column, this ratio is most easily adjusted by changing the strength of the mobile phase, which tunes the solute-column affinity. While these interactions occur on the molecular level, the entire process is analogous to classic zone migration\(^{17}\).

#### 2.4.1. HPLC column variables

As mentioned above, migration of a component through an HPLC column depends on the component velocity within the mobile phase. Since retained components spend some time interacting with the stationary phase, they can only travel at a fractional rate of the eluent flow. Components which have no interaction with the stationary phase elute in the minimal elution volume, \(V_o\). The solute retention is expressed mathematically by:

\[
V_r = V_o + K V_s \tag{1}
\]

where \(V_r\) is the experimentally measured elution volume of the retained component, and \(V_s\) is the stationary phase volume. The constant \(K\) indicates the extent of
stationary phase interaction, defined as the ratio of component concentrations in the stationary and mobile phases \((C_s/C_m)\). When the components are charged, a distribution ratio, \(D\), is used in place of \(K\).

**The retention factor** \((k')\): The relative amount of each component in each of the two phases, and \(k'\) is calculated from chromatographic results using:

\[
k' = \frac{V_r - V_o}{V_o}
\]

Optimum \(k'\) for less complex separations lies between 1.5 and 4. Values less than 1.5 indicate little retention, while values exceeding 4 show too much retention. Excessive retention does not contribute to better separations, instead it tends to cause longer analysis time and peak broadening. In more complex separations, the acceptable range is often taken as \(1 < k' < 10\).

**The number of theoretical plates** \((N)\) is used as a measure of separation efficiency which describes the potential separation capacities of the chromatographic system. Theoretical plates can be thought as the number of interaction instances that a solute experiences as it passes through the separation column. The more frequently a compound is able to move into and out of the stationary phase during a given separation, the higher the efficiency.

There are several ways to calculate \(N\) from the chromatogram. The most simple approach utilizes the retention time \((t_r)\) taken from the peak apex (or more correctly center of gravity) and the baseline peak width \((w)\), which found as the distance between intersections of the tangents at the inflexion points on each side of the peak and the baseline:

\[
N = \sigma^2 = 16\left(\frac{t_r}{w}\right)^2
\]

A Gaussian peak shape is assumed and the height equivalent to a theoretical plate, \(H\), therefore equals the variance of the peak, \(\sigma^2\) divided by the column length, \(L\):

\[
H = \sigma^2/L
\]

In order to compare the efficiencies of different length columns, the height equivalent to a theoretical plate, \(H\) (or \(HETP\)), is used instead of \(N\).

**The selectivity** \((\alpha)\) shows the extent of separation of two different components on a column and is calculated from:

\[
\alpha = \frac{k'_1}{k'_2}
\]
Where \( k'_1 \) and \( k'_2 \) are the retention factors of the first and second components of the solute pair being considered. Values of \( \alpha \) are therefore always > 1, the larger the better the separation. When \( \alpha \) approaches unity, very efficient separation is needed to resolve the components into fully separated peaks.

**The resolution** \( (R) \) takes into account the effects of both efficiency \( (N) \) and selectivity \( (\alpha) \) on a separation is given by the resolution, \( R \), is estimated from the chromatogram using:

\[
R = \frac{\Delta t}{(w_1+w_2)^{1/2}} = \frac{1}{2(\alpha+1)(1+k')} \frac{N^2(\alpha-1)k'}{2(\alpha+1)(1+k')}
\]

Where \( \Delta t \) is the difference in retention times between the two compounds being considered, \( w \) is the widths of peaks 1 and 2 at their bases (Figure 3), \( k' \) is the average retention factor\([k'=(k'_1+k'_2)/2]\), \( \alpha \) is the selectivity, and \( N \) is the average number of theoretical plates.

**Figure 3.** Resolution determined by the peak width and distance between two chromatographic peaks.

In liquid chromatography, optimizing the separations through varying resolution, speed, and sample size can be done. One of the factors is optimized at the expense of the remaining two. The choice of conditions depends on which factor is most important for a given situation.
3. Hydrophilic Interaction Chromatography (HILIC)

3.1. Introduction

HILIC is an HPLC mode that is particularly well suited as alternative to RP-HPLC and ion exchange chromatography for separation of neutral and polar compounds that show no or minimal retention in those modes. The origins of HILIC are often traced back from the separation of sugars\(^1\) in which a Bondapak AX/Corasil column with 37-50 µm particle size was used as a stationary phase, with eluents composed of ethyl acetate, 2-propanol, and water. Better resolution was obtained with acetonitrile-water mixture with acetonitrile content from 75-90% (v/v) as elution solvents. Since 1990, the number of publications on HILIC has increased substantially as outlined in the review by Hemström and Irgum\(^3\). Like NPC, HILIC was initially practiced on neat, underivatized silica or silica furnished with “traditional” polar bonded chemistries such as 3-aminopropyl, 2,3-hydroxypropyl, or 3-cyanopropyl, using mobile phase quite similar to those employed in RP-HPLC, i.e., partly aqueous mobile phases. What made HILIC eluents differ from RP-HPLC counterparts is that they are typically richer in organic component, which is usually acetonitrile due to its combination of 100% water miscibility and absence of hydrogen bonding properties.

The separation mechanism in HILIC is more complicated than that in RP-HPLC.\(^{11}\) The initially proposed partitioning mechanism in liquid chromatography was summarized in the 1941 paper by Martin and Synge.\(^{18}\) They performed separations of amino acids on silica columns, using water-saturated chloroform as mobile phase, and attributed the observed separations to partitioning between the bulk mobile phase and a water layer on the surface of the stationary phase. The presence of a water layer on the surface of neat silica under HILIC conditions has recently been demonstrated\(^{19}\). There are also several extensive reviews of both stationary phases and retention mechanisms in HILIC.\(^{3,7-11,20}\) Recent research on the solute retention mechanism in HILIC has showed that the dominant retention mechanism is a mixed mode, in which hydrophilic partitioning and ionic exchange interaction are involved\(^{3-7}\). Under appropriate experimental conditions, even hydrophobic interactions may be involved\(^{21-25}\). A distinct advantage of HILIC is that the selectivity is essentially opposite to RP-HPLC; very hydrophilic compounds with poor or non-retention in RP-HPLC are often well retained in HILIC, while in NPC mode these polar solutes dissolve only poorly in the non-aqueous mobile phases. Additional advantages of the acetonitrile-rich partly
aqueous eluent used in HILIC is a low back pressure due to the low mobile phase viscosity, which is coupled with high sensitivity in electrospray mass spectrometry (ESI-MS). Recent works have also reported good peak shapes and high loadability for protonated basic compounds, with flatter van Deemter curves at high mobile phase velocity. HILIC therefore continues to find applications in new fields, e.g., in metabolomics and proteomics.

3.2. Separation materials for HILIC

As mentioned above, only a few columns dedicatedly synthesized for HILIC were available in the 1990s and early 2000s. Most of the early HILIC separations were therefore performed on columns synthesized for normal phase chromatography (e.g., 3-aminopropyl, 3-cyanopropyl, and 2,3-hydroxypropyl bonded phases), in addition to underivatized silica. Phases that have been commercially available practically since the inception of HILIC are the poly(succinimide)-based silica coatings devised by Alpert and their neutral sibling PolyHYDROXYETHYL A. The basic range of HILIC columns includes plain silica, as well as silicas derivatized with neutral polar, ion-exchange, and zwitterionic ligands. Readers will find state-of-the-art knowledge on stationary phases dedicated for HILIC in recent reviews covering the understanding of HILIC mechanism and the development of new polar stationary phases, many of which are now commercialized. HILIC has created such a demand for in new columns, that a wide variety of new phases have been developed. Below follows a summary of stationary phases with unique surface chemistries based on silica and polymer supports, both in particulate or monolithic formats.

3.2.1. Silica and polymer-based particulate stationary phases

3.2.1.1. Amine stationary phases

Amino-bonded silica phases are still very attractive in HILIC mode because of the usefulness in application involving separation of sugars, amino acids, carboxylic acids, peptides, surfactants, and pharmaceuticals. A drawback of the traditional 3-aminopropyl bonded silica is that the amino group is prone to form Schiff bases with carbonyl compounds (aldehydes and acetones). Stationary phases containing secondary or tertiary amine groups have therefore been developed. For example, Chen et al. introduced a bidentate amino stationary phase dedicatedly synthesized for HILIC by Michael addition of methyl acrylate to
aminopropyl silica, followed by amidation of the methyl esters with ethylenediamine; synthetic route is shown in Figure 4. This novel primary/tertiary amino/amide phase (SG-EDA) demonstrated stronger retention and a different selectivity for organic acids compared to the classic aminopropyl silica phase (SG-NH₂) (Figure 5).

**Figure 4.** Synthetic route of traditional primary amino and bidentate amino stationary phases.³¹ Reprinted with permission from Taylor & Francis Group, LLC publishers.

Another weak anionic exchange amino stationary phase was recently introduced by Lämmerhofer et al.³²-³⁴ The structure of this stationary phase is presented in Figure 6, where a distal weak anion-exchange-type quinuclidine moiety is linked to the silica via an amide linkage, a hydrophobic alkyl spacer, and a thioether. The phase can thus be characterized as a mixed mode weak anion exchange (WAX) functionality with mild hydrophobic character, where excess polarity is derived from the dual polar embedded groups (the amide and thioether linkages). This mixed mode separation material has indeed proven to possess a multi-modal retention mechanism including attractive or repulsive electrostatic interactions.

**Figure 5.** Separation of organic acid mixture on SG-EDA (a) and SG-NH₂ (b). Conditions: 70/30 % acetonitrile/5 mM ammonium formate, pH 5.2; flow rate, 1 mL/min; injection volume, 20 µL; UV 254 nm. (1) Sorbic acid, (2) o-aminobenzoic acid, (3) benzoic acid, (4) p-hydroxybenzoic acid. Reprinted with permission from Taylor & Francis Group, LLC publishers [31].
overlaid on hydrophobic and/or hydrophilic interactions for peptide, metabolites, mycotoxins, and sugar phosphate separations.\textsuperscript{33,34}

![Figure 6. Chemical structure of mixed-mode reversed-phase/WAX phases. Reprinted with permission from Springer [34].]

Tetrazole based stationary phases were introduced for HILIC separations by Wei \textit{et al.}\textsuperscript{35,36} The tetrazole motif has been used as a bioisosteric replacement for carboxylic acid due to its aqueous $pK_a$ value being similar to carboxylic acids. The tetrazole group can therefore be used as ligand in weak cation exchange (WCX) mode. The chemical structures of tetrazole functionalized stationary phases based on silica\textsuperscript{35} and on glycidylmethacrylate-copoly-ethylene dimethacrylate polymeric particles\textsuperscript{36} are shown in Figures 7 and 8, respectively. A HILIC mechanism was observed on these phases at higher content (85 volume-%) of acetonitrile in the mobile phase and was proven to be mainly due to surface adsorption mechanism using theoretical models. Poly(vinyltetrazole)-rafted polymeric particles were investigated for the separation of proteins in weak cation exchange (WCX) mode and nucleosides in HILIC mode with decent efficiency.

![Figure 7. Scheme of tetrazole functionalized silica prepared by nitrile-modified silica by an ammonium catalyzed (3+2) azide-nitrile cycloaddition reaction. Reprinted with permission from Springer [35].]
3.2.1.2. Di-(poly)-ol phases

Diol phases are usually prepared by covalent bonding of neutral, hydrophilic 2,3-dihydroxyalkyl groups to silica. This ligand has a relatively high polarity and also offers hydrogen-bond donor- and acceptor properties. Diol phases contain intentionally ionizable groups other than non-reacted residual silanols, which can be partially blocked by a silylating reagent.10 Diol groups bonded to silica via a propyl anchor can be synthesized by bonding glycidoxypropyltrimethoxysilane to the silica gel surface, followed by acidic hydrolysis of the epoxy groups37. Alternatively, the diol functionality can be separated from the silica surface by an alkyl chain (undecyl; C₁₁) by attaching undecyl-1,2-diol ligands onto silica gel.38 The latter phase showed a dual RP/HILIC retention mechanism for the analysis of non-ionic ethoxylated surfactants due to the combination of a hydrophobic alkyl chain and a terminal polar diol group.38A polymeric and cross-linked diol silica phase with the commercial name Luna HILIC was introduced by Phenomenex and is said to have increased stability against hydrolysis, stronger hydrophobic interactions, and better peak shape and resolution compared to non-cross-linked diol-silica phases,39 irrespective of its rather low retention of polar compounds under HILIC separations.6
A polyhydroxy-type tentacle phase was prepared by grafted sorbitol methacrylate onto silica particles by free radical polymerization (the synthetic scheme is shown in Figure 9). This highly hydrophilic polyol phase exhibited a markedly different selectivity from that of and neat silica and several commercially available columns when subjected to evaluation in HILIC mode.40

![Figure 9. Graft polymerization of sorbitol methacrylate from the surface of silica particles.](image)

Reprinted with permission from WILEY-VCH, Weinheim [40].

### 3.2.1.3. Polysaccharide phases

Polysaccharide phases including mono- and disaccharides (*e.g.* glucose, fructose, and maltose) and oligo-/poly-saccharides (*e.g.* cyclodextrins and cyclofructans) have established a HILIC stationary phase group of their own, owing to their high polarity and enantiopurity that enables separation of chiral compounds in HILIC mode.10 Synthesis of polysaccharide stationary phases via ‘click chemistry’ has been successfully demonstrated and provides a facile and efficient novel strategy for covalent bonding of functionalities onto HPLC grade silica beads. A review of click chemistry application for preparation of separation materials for HPLC was recently published by Chu et al.41 The ‘click chemistry’ used in these preparation is based on copper-catalyzed azide-alkyne cycloaddition. A variety of mono- and disaccharide covalently bonded silicas were also synthesized via ‘click chemistry’ in which sugar alkynes are covalently coupled to the azido-activated silica gel surface in the presence of a copper catalyst for used in for HILIC.42-45

A β-cyclodextrin (β-CD) phase was prepared via ‘click chemistry’ by bonding the azide-modified β-cyclodextrin onto the surface of alkyne-modified silica particles, and was investigated in HILIC mode for the separation of nucleosides, organic acids and alkaloids.46,47 Due to the chiral recognition properties under HILIC conditions, click β-cyclodextrin phase has also been applied to separation of some flavones and isoflavones, which co-eluted under RP-HPLC conditions.48,49 Liang et al. applied a β-CD column for two-dimensional liquid chromatography (2D-HPLC) for analysis of polar components in a traditional Chinese medicine.50

Another interesting saccharide derivative stationary phase synthesized via ‘click chemistry’ for use in HILIC is based on glycosyl amino acid functionalized silica.51
The reaction scheme for the synthesis of this stationary phase is outlined in Figure 10, in which the prepared azide N₃-glycosyl-D-phenylglycine (indicated as ‘4’ in Figure 10) is reacted with alkyne modified silica in the presence of a copper catalyst. Chromatographic tests showed that this new type of separation material possessed good HILIC properties. Nucleosides, bases, and polar organic acids could be separated in a simple salt-free eluent composition, containing only acetonitrile in combination with water. The same model compounds could not be separated on a commercial HILIC column (Waters Atlantis HILIC Silica) under identical conditions. Due to its hydrophilic characteristics and structure similar to glycopeptides, this phase also provides glycopeptide enrichment characteristics.51

Conventional free radical polymerization was also used to synthesize a mixed-sulfated/methacryloyl polysaccharide derivative onto the surface of porous silica particles functionalized with vinyl groups in an ionic liquid by Li et al.52 This mixed mode phase showed both hydrophilic interaction (HILIC) and ‘per aqueous’ liquid chromatography (PALC) characteristics. In “PALC mode”, which was also called reversed HILIC because it uses highly aqueous (90-100 volume-%) eluents, the new polysulfate/saccharide column had weaker retention for weak polar and non-polar compounds, but showed stronger retention for highly polar compounds compared with C₁₈ columns, with a retention pattern as in HILIC mode for polar compounds.52

Figure 10. Reaction scheme of click glycosyl phenyl glycine on silica beads. 4 is azide N₃-glycosyl-D-phenylglycine. Reproduced by permission from Elsevier [51].

Lindner et al. also introduced a new way of synthesizing saccharide-based silica phases by applying non-enzymatic browning (the Maillard reaction) to reducing sugars attached to amino-functionalized silica surfaces by Schiff base formation (the reaction scheme is shown in Figure 11.53 The authors provided a detailed evaluation of this “Chocolate” phase (the brown circles in the Figure 11 represents the as yet structurally less defined “Chocolate” ligands) with five different sets of compounds of different polarity and charges, verifying that the material is indeed
useful in HILIC mode with deficient hydrophobic interactions. In terms of retention mechanism, the new “Chocolate” phases behaved as mixed mode stationary phases when used in the HILIC mode, *i.e.* adsorption (including ionic interactions) and partition phenomena were involved.\(^{53}\)

Derivative sulfonate substituted cyclic oligosaccharide bonded silica based on cyclofructans (CFs) was introduced by Armstrong *et al.*\(^{54}\)Cyclofructans (CFs) are cyclic carbohydrates that consist of six or more β-(2→1) linked D-fructofuranose units, in which each fructofuranose unit contains one primary hydroxyl group and two secondary hydroxyl groups that contribute to making this molecule highly hydrophilic.\(^{55}\) A large variety of polar compounds (β-blockers, xanthines, organic acids, nucleic acid bases, nucleosides, maltooligosaccharides, water soluble vitamins, and amino acids) were evaluated on this new column with varying sulfonate substitution and compared with a commercially available ZIC-HILIC column.

### 3.2.1.4. Zwitterionic stationary phases

Zwitterionic sulfoalkylbetaine and phosphorylcholine functionalized silica gel and polymeric supports introduced by Irgum’s group represent the two main classes of zwitterionic type stationary phases, which are nowadays widely used in HILIC separation applications.\(^{56-60}\) These phases are all synthesized by polymerization of a zwitterionic monomer onto the solid supports, and have been further developed into the commercial phases ZIC-HILIC (polysulfoalkylbetaine on silica particles), ZIC-pHILIC (polysulfoalkylbetaine on polymeric particles) and ZIC-cHILIC (polyphosphorylcholine on silica particles).

Recently, Qui *et al.* introduced a new zwitterion type stationary phase based on 3-*P,P*-diphenylphosphoniumpropylsulfonate covalently bonded silica gel (structure in Figure 12) at low (8 %; **L-ZI**) and high (12 %; **H-ZI**) degree of functionalization, assessed by carbon loading according to elemental analysis, as well as a variant of the H-ZI material, where residual unreacted silanols were end-capped by reaction with trimethylchlorosilane (14 % C; **EC-ZI**).\(^{61}\) The H-ZI phase showed a selectivity
for separations of β-blockers, water soluble vitamins, nucleic acid bases, and nucleosides in HILIC mode that was different from the commercially available ZIC-HILIC material used as benchmarks. This selectivity difference is rationalized by π-π interaction on H-ZI due to the different cationic moieties on the stationary phases (diaromatic quaternary phosphonium on H-ZI vs. dialiphatic quaternary ammonium on ZIC-HILIC). They also showed that the new H-ZI phase gave better resolution than the ZIC-HILIC phase for some of the tested polar compounds even though one should keep in mind that the 100 Å pore size of the silica used as substrate in the synthesis of the H-ZI phase was smaller than that of the ZIC-HILIC column chosen as benchmark (200 Å). Further, a major difference is that the H-ZI material is functionalized by a monomeric ligand directly attached to the silica, whereas the ZIC-HILIC has the functionalities added as a grafted polymeric layer. This comparison may therefore be a bit too limp because this pore size difference could lead to a substantial difference in the surface coverage and hence selectivity and separation resolution. The results also showed lower separation capability on the end-capped EC-ZI column compared to non-end-capped precursor H-ZI, which reveals that the residual silanols play an active role in the retention mechanism.

Another zwitterionic stationary phase, **Click TE-Cys**, was recently synthesized by Shen et al.\textsuperscript{62}, based on covalent bonding of cysteine to vinyl modified silica via a metal-free ‘thiol-ene’ click reaction, for intended use in HILIC (Figure 13). By this attachment chemistry, the positively and negatively charged groups are distributed uniformly and parallel to the surface of the silica gel. This phase is particularly interesting since it has a combination of WAX and WCX functionality, which can be
tuned by changing the pH of the eluent. This charge switch (the phase pI) takes place at a pH slightly above 4 in aqueous solution, evident from the zeta potential measurements which showed values (measured in water) of +3.0 mV at pH 4.0, and −8.4 mV at pH 5.0. The Click TE-Cys phase provided good separation of oligosaccharides, peptides, and basic compounds with improved peak shapes compared with C18RP-HPLC. It also proved capable of enriching glycopeptides under HILIC conditions. However, the short communication where the Click TE-Cys phase is described has no detailed investigations of salt, organic modifiers, and pH effects on the retention pattern of solutes in HILIC mode.

![Image](image.png)

**Figure 13.** Synthesis of the Click TE-Cys zwitterionic functionalized silica. Reagents/conditions: (i) vinyl trichlorosilane, toluene, RT; (ii) cysteine, AIBN, MeOH:H2O (1:2), 65 °C, N2 atmosphere. Reprinted with permission from The Royal Society of Chemistry [62].

The same group has more recently prepared another pH-dependent zwitterionic ε-amino tethered lysine phase (CLSP) with WAX/WCX properties for intended use in HILIC by covalently bonding L-azido lysine via click chemistry to a silica that had been furnished with terminal alkyne groups according to the synthetic route in Figure 14. The CLSP phase provided better retention and higher efficiency for nucleosides and bases, as well as organic acids compared with the commercially available Atlantics HILIC silica and a ‘traditionally’ prepared lysine functionalized silica phase in HILIC mode. The authors also applied the CLSP phase for separation of highly polar cephalosporin and carbapenem antibiotics that were retained in C18RP-HPLC only at water concentrations > 94%.
Figure 14. The preparation of an ε-amino tethered zwitterionic lysine phase (CLSP) phase by click chemistry. Reproduced with permission from Elsevier[63]

3.2.1.5. Thiol and sulfoxide stationary phases

A family of non-ionic silica-based phases was introduced by Wu et al.\textsuperscript{64} by reaction of 2-mercaptoethanol and 1-thioglycerol onto vinylized silica to yield new phases designated as ME and TG (Figure 15). These ligands were then subjected to oxidation with hydrogen peroxide in aqueous medium to yield packings MEO and TGO, where the embedded thioether linkages had been oxidized to sulfoxides. When compared to three commercial diol phases, all these phases showed a combination of adsorption (NPC type) and partitioning (HILIC type) retention mechanism for nucleobases and nucleosides with HILIC mode eluents. Among the synthesized phases, the MEO and TGO phases were more hydrophilic than ME and TG, and this was attributed to a higher polarity caused by oxidative conversion of the thioethers to sulfoxides. Silanophilic activity was also noted for all four synthesized phases, more pronounced in the thioether-linked than in the sulfoxide-linked materials. In RP-HPLC mode, the packings also showed shape selectivity for π-aromatic compounds of different sterical structures due to sulfur atom.
3.2.1.6. Mixed-mode phases

Simultaneously incorporation of different functionalities onto silica to establish intentional mixed-mode stationary phases may give advantages in both RP-HPLC and HILIC modes. Ma et al.\textsuperscript{65} prepared a pH-responsive polymer-grafted silica by a free radical “grafting from” polymerization of acrylic acid and butyl acrylate in di-oxane, initiated by 4,4’-azobis(4-cyanovinyl)acid chloride coupled to 3-aminopropyl silica. The polymer grafted silica was pH-responsive, i.e., the hydrophobic surface properties are more pronounced at lower pH, whereas a more hydrophilic character is developed at higher pH. Separations of sulfonamides and soybean isoflavones were carried out in RPLC mode and the separation of some nucleotides was achieved in HILIC mode. However, the packing prepared by this uncontrolled free radical polymerization gave low column efficiency and peak tailing.

Another interesting mixed-mode silica-based stationary phase was obtained by immobilization onto aminopropyl silica via amide-bond formation \textit{humic acid}, the collective name given to the complex polymeric substances bearing hydrophobic, hydrophilic, aromatic, and ionic functionalities, produced by the decay of dead organic matter (Figure 16).
The intended use of this rather vaguely characterized phase is multimodal HPLC separations of nucleosides and nucleobases.\textsuperscript{66a} This stationary phase showed a RPLC/HILIC mixed-mode behavior with plots of retention factors ($k'$) vs. volume percentage of organic modifier exhibiting a U-shaped curve.

Another multi-mode poly(ionic liquid)-grafted phase has been prepared based on polymerizing the ionic liquid monomer, 1-(2-acryloyloxyundecyl)-3-methylimidazoliumbromide ([mC\textsubscript{11}C\textsubscript{1}Im]Br) onto silica particles modified with 3-mercaptopropylgroups by a surface-initiated radical chain-transfer reaction (Figure 17).\textsuperscript{67} Compared with commonly used ODS columns, the mixed mode ionic liquid phase showed considerably higher molecular-planarity selectivity towards polycyclic aromatic hydrocarbon isomers, anion-exchange ability for inorganic anions, and a HILIC type separation pattern for nucleosides and nucleic bases.

**Figure 16.** Representative structure of a humic acid fragment, showing the wide variety of components including quinones, phenolics, catechols, and carbohydrate moieties. Reproduced with permission from John Wiley & Sons, New York [66b].

**Figure 17.** Synthesis of mixed-mode polymeric ionic liquid grafted silica particles. Reproduced with permission from The Royal Society of Chemistry [67].
Among the more esoteric phases evaluated in HILIC is a metallacarborane covalently bonded silica according the reaction scheme shown in Figure 18. This column expressed hydrophobic and zwitterionic properties and hence showed a mixed-mode retention mechanism, in which the reversed phase properties were dominant besides hydrophilic partitioning mechanism. In HILIC mode, the phase showed hydrophilic interactions with some nucleic bases in an acetonitrile/water mixture in the range of 70-95% acetonitrile in aqueous buffer.

![Figure 18. Reaction scheme for preparation of metallacarborane covalently bonded silica. Reprinted with permission from Elsevier[68]](image)

### 3.2.1.7. Hybrid silica-organic stationary phases

Bridged ethylene hybrid (BEH; Figure 19) and amide-bonded BEH (BEH-Amide) silica particles of 1.7 µm particle diameter were assessed for separations in HILIC mode by Grumbach et al. Both materials showed reasonable retention in at high mobile phase organic modifier concentration (ACN > 80%), especially BEH-Amide packing was used for separation of polar basic pteridines with improved retention and peak shape compared to the BEH packing. These small particle size phases give high throughput and sensitivity compared to 3 µm silica in ESI-MS. Since nearly one-third of the surface silanols are removed in BEH particles, both hybrid materials have an improved chemical stability and performance. HILIC retention mechanisms are a complex combination of partitioning, adsorption and secondary interactions, which have been demonstrated on both BEH materials.
3.2.2. Silica and Polymer-based Monolithic stationary phases

In contrast to columns packed with porous (or nonporous) particles, monolithic stationary phases with continuous flow-through separation media provide higher column permeability which enables faster separations under moderate operation pressure. There are two major types of monolithic stationary phases, silica-based monoliths with a bimodal macro-/mesopore distribution and organic polymer-based monoliths where the pore size distribution is usually less well defined.10

3.2.2.1. Silica-based monolith stationary phases

Monolithic silica was first introduced in 1996 by Nakanishi and Tanaka.71 These materials are characterized by a pore size distribution with intra-monolithic flow-through pores, typically \( \approx 2 \, \mu m \) average diameter, and skeletal mesopores ranging from 2 to 50 nm in diameter. Silica monoliths are particularly well suited for fast separations of small molecules and peptides,72 and have been commercially available from Merck (Darmstadt, Germany) under the brand name Chromolith for more than a decade. Pack and Risley73 evaluated a Chromolith Si column with neat silica surface functionality for the separation of a rather odd set of solutes, namely separation of polar drugs along with their counterions (Li\(^+\), Na\(^+\), and K\(^+\)) in pharmaceutical preparations. Although HILIC-like conditions were used (60–80% acetonitrile), the separations were likely more based on ion exchange than on a HILIC mechanism.

Functionalization of monolithic silica columns with different functionalities for HILIC applications is well developed. Polymer-coated capillary silica monoliths prepared by direct on-column polymerization of various vinylic monomers on the monolithic silica surfaces modified with 3-(methacryloxypropyl)trialkoxyisilane or 3-(methacrylamidopropyl)trialkoxyisilane is a commonly used route. For example,
Toru et al.\textsuperscript{74} used this approach to synthesize an acrylamide functionalized silica monolith capillary. This phase showed HILIC mode retention characteristics with three times greater permeability and slightly higher column efficiency compared to a commercially available amide-type HILIC column with 5 μm particles.\textsuperscript{74,75} The same group later introduced other polymer-coated monolithic silica columns including acrylic acid for weak anion exchange/HILIC\textsuperscript{76} and 3-diethylamino-2-hydroxypropyl methacrylate and p-styrenesulfonic acid for cation exchange chromatography\textsuperscript{77}.

Jia et al.\textsuperscript{78} introduced a cationic monolithic HILIC stationary phase for capillary LC by attaching to a silica monolith skeleton carbodiimide activated \textit{carboxymethyl chitosan} (CMCH), which is a modified biopolymer prepared by deacetylation and carboxymethylation of \textit{chitin}, a structural polymer supporting the exoskeletons of crustaceans (Figure 20). The amino-and hydroxyl-moieties of CMCH functioned both as weak anion exchange sites and polar retention promoters. However, this CMCH functionalized monolithic silica column showed a lower column efficiency compared to the poly(acrylic acid) monolithic columns prepared by Toru et al.\textsuperscript{76} The group of Jia has also shown an \textit{N}-methylimidazolium grafted monolithic silica capillary column for separation of inorganic anions, aromatic acids, nucleotides, polycyclic aromatic hydrocarbons, alkylbenzenes, and phenols.\textsuperscript{79} The retention mechanisms appeared to involve a variety of interaction modes including anion-exchange, hydrophilic, π–π, dipole–dipole, and hydrophobic interactions.

![Figure 20](image_url). Reactions used to synthesize carboxymethyl chitosan functionalized silica monolith. Reproduced with permission from Elsevier\textsuperscript{[78]}. 

25
Very recently, a new type of zwitterionic organic-silica hybrid monolithic capillary columns aimed at HILIC separation was synthesized based on the monomers [2-(methacryloyloxy)-ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (MSA) or 2-methacryloyloxyethyl phosphorylcholine (MPC) and 3-methacryloxypropyltrimethoxysilane (γ-MAPS) as cross-linker via a single-step thermal polymerization, as outlined in Scheme 1. The hydrolysis/condensation of the alkoxysilane and the free radical polymerization of the vinylic entities happen simultaneously in this approach to form a hybrid organically modified silica (Ormosil) monolithic capillary column, which was used for the separation of polar compounds as well as small peptides and tryptic digest of bovine serum albumin (BSA) by capillary hydrophilic-interaction chromatography tandem mass spectrometry (HILIC-cLC-MS/MS). A typical HILIC retention mechanism was observed at higher organic solvent contents (> 50 % ACN).

Scheme 1. A single-step thermal-treatment "one-pot" approach for the preparation of organic-silica hybrid capillary monolithic columns. Reprinted with permission from ACS [80].

3.2.2.2. Organic polymer-based monolith stationary phases

Polymeric monoliths as they exist today were developed independently by three different labs in the late 1980s, led by Hjertén, Svec, and Tennikova. Like their silica counterparts, organic monolithic polymer stationary phases possess flow-through pores in an inter-adhered polymer globule structure, but most organic monoliths contain any appreciable amount of mesopores (if any). In the absence of small pores in the polymer skeleton, mass transfer to the stationary phase is mainly due to convection rather than diffusion. This makes organic monoliths well suited for fast gradient separations of large molecules, especially proteins, since separation of large molecules can be carried out at limited column efficiency because of very steep elution curves (k’ vs. strong eluent content). These materials generally show rather poor efficiency for the separation of small molecules, which is attributed to the restricted fluid transport in the stagnant mobile phase inside
the extensive micropore system, into which small molecules can permeate, but only slowly diffuse back.\textsuperscript{85}

The situation described above is somewhat alleviated in capillary electrochromatography (CEC), where the eluent flow is established primarily by electro osmosis. As a consequence, a variety of zwitterion-based monolithic polymer columns have been developed for CEC by thermal co-polymerization of 2-methacryloyloxyethyl phosphorylcholine (MPC) and ethylene dimethacrylate (EDMA) for HILIC\textsuperscript{86} or by photo-initiated copolymerization of $N,N$-dimethyl-$N$-methacryloxyethyl-$N$-(3-sulfopropyl)-ammonium betaine (SPE) crosslinked by either ethylene dimethacrylate (EDMA) for cation-exchange LC of proteins\textsuperscript{87} or poly(ethylene glycol) diacrylate for HILIC\textsuperscript{88}. Jiang \textit{et al.} synthesized a series of zwitterionic polymeric monolithic columns for HILIC by thermal-initiated copolymerization.\textsuperscript{89-91}

Monolithic columns with quaternary amino groups affording both hydrophilic and electrostatic interaction has been prepared by copolymerization of 2-(methacryloyloxy)ethyltrimethylammoniummethyl sulfate and pentaerythritol triacrylate for HILIC mode in mobile phases with acetonitrile content > 20 \%.\textsuperscript{92} Peak tailing of basic compounds was avoided and efficient separation of benzoic acid derivatives was obtained.

Wrapping up this section, it is worth mentioning that Jandera \textit{et al.} demonstrated advantages of the hybrid inter-particle monolithic columns in terms of chromatographic separation efficiency and selectivity in both reversed phase and HILIC modes. The results showed that separation efficiency and selectivity were in between purely particle-packed and purely monolithic columns.\textsuperscript{93}

3.2.3. Metal oxide-based stationary phases

Despite the numerous advantages of silica-based stationary phases (\textit{e.g.} absence of artificial peaks resulting from column bleeding in LC/MS), silica as a substrate still suffers from limited pH and thermal stability. This drawback can be overcome by replacing silica with hydrolytically more stable metal oxides such as zirconia, titania, and alumina. These materials are more stable at extreme pH and are able to withstand high temperature.\textsuperscript{94} Hence, these metal oxide-based phases allow the analysis of strong acids or bases in their non-ionized form, and utilization of separation at elevated temperature.\textsuperscript{94} Several publications have demonstrated the utility of titania-based stationary phases in HILIC mode. On a bare titania, Lucy \textit{et al.} demonstrated the HILIC behavior of nucleotides\textsuperscript{95} and carboxylic acids.\textsuperscript{96}
Randon and co-workers have separated xanthines\textsuperscript{97} and β-blockers\textsuperscript{98} on titania in HILIC mode and also described the use of titania monoliths for extraction of nucleotides in HILIC mode\textsuperscript{99}. The same group have described the separation of selected xanthine derivatives on capillary zirconia monolithic columns (zirconia monoliths and zirconia coated on silica monoliths) in HILIC mode.\textsuperscript{100} Klimes \textit{et al.} investigated zirconia stationary phases and found that both native zirconia and polybutadiene coated zirconia phases retain polar acidic compounds in HILIC mode.\textsuperscript{94} Interestingly, tin(IV) oxide (SnO\textsubscript{2}, stannia) microspheres were tested as a new type of metal oxide material for phosphopeptide enrichment.\textsuperscript{101}

In general, these metal oxides-based materials have an amphoteric character and Lewis acid sites on the surface. Therefore, they can work as either anion or cation exchangers depending on the mobile phase pH. This lends chromatographic metal oxides-based stationary phases properties that are quite different from those of the more commonly used silicon oxide (silica).\textsuperscript{102} Multiple retention mechanisms are assumed to be involved including electrostatic repulsion, ligand exchange, or hydrophilic partitioning depending on the eluent conditions. However, these papers also identify a lack of methods to characterize the retention mechanism on these metal-oxides-based stationary phases under different HPLC modes.

4. \textit{From “ideas” to “experiments…”}

The overview given above on stationary phases that have been synthesized for use in HILIC mode shows that most silica-based bonded polar phases are synthesized by conventional silylation chemistry producing a single layer of ligands with polar functional groups onto the silica surface. Only a limited number of silica-based HILIC phases are synthesized with the aim of forming polymeric functional groups. Among these are the first HILIC phases prepared, the poly(succinimide) based phases of Alpert and the polyzwitterion silica-based phases from the group of Irgum\textsuperscript{3}. According to the commonly agreed retention mechanism in HILIC, as discussed above, the retention of polar solutes to be separated by a hydrophilic partitioning mechanism will increase with the volume (and hence thickness) of a water-swollen dressing layer attached to the silica surface. Grafted polymer layers seem to be uniquely suitable for this purpose\textsuperscript{5}, and these hydrogel coatings can be custom designed to contribute to specific interactions involved in polar interactions, such as hydrogen bonding, dipole-dipole, and also electrostatic interactions.
With this in mind, our interest focused on synthesizing silicas with pore surfaces grafted by polyhydroxy- and amide-based hydrogel layers. An obvious candidate monomer for these efforts was \(N\)-[tris(hydroxymethyl)methyl]acrylamide (TRIS-acrylamide) (Figure 21). A stationary phase grafted with this monomer contains no ionizable groups other than residual unreacted silanol groups (if any). The phase should then ideally neutral and the retention of neutral polar solutes on the TRIS-Acrylamide functionalized silica phase is expected to be governed practically solely by hydrophilic partitioning. Single mode retention mechanisms can in HPLC be preferable to a mixed-mode mechanism since higher separation efficiency can be obtained due to faster solute mass transfer, \textit{i.e.}, the equilibrium between the mobile and stationary phases can be established more rapidly because of intricate surface-oriented bonds need not be formed and broken.

![Figure 21. Chemical structure of \(N\)-[Tris(hydroxymethyl)methyl]acrylamide (TRIS-acrylamide).](image)

While carrying out the synthesis of the TRIS-Acrylamide based material, an idea to synthesize an analog based on 2-amino-2-hydroxymethyl-1,3-propanediol or tris(hydroxymethyl)aminomethane (TRIS) (Figure 22) popped up in my mind. The polyhydroxy-and amine-based material, namely TRIS-WAX (\textbf{Paper I}) is expected to retain a high HILIC mode retention similar to the tris(hydroxyethyl) moiety used for preparing the other polyhydroxy- and amide-based material namely TRIS-Amide (\textbf{Paper II}), but would show a radically different selectivity due to the secondary amine functionalities accounting for weak anion exchange (WAX) property, which is devoid in the TRIS-Amide material. Characterization of their actual ability to separate typical HILIC probes are presented in \textbf{Papers I and II} and detailed studies of their interaction modes for different model compounds relative to a few selected commercially available HILIC columns that are also based on polymer functionalized silica were further investigated in \textbf{Paper III}. 
With the aim of enhancing the chemical stability of polar polymeric ligands that are covalently bonded to silica when using aqueous HILIC eluents, materials with a crosslinked layer close to the silica surface, with a tentacle layer on top are of interest. The process is realized by surface-initiated controlled polymerization. In Paper IV, we introduced an example of this graft-block copolymer functionalized silica based on polar crosslinking monomer $N,N'$-methylenebisacrylamide (BIS) (Figure 23) as the crosslinker and 2-methacyryloyloxyethyl phosphorylcholine (MPC) (Figure 24) as the second monomer to graft on top of the BIS layer. The resulting material was compared with a commercially available zwitterionic phosphorylcholine phase based on the MPC monomer, namely ZIC-chHILIC from Merck.

Throughout the thesis, porous silica particles (Kromasil) of 5 µm particle size and 200 Å pore size were used as the starting substrates for functionalization and polymerizations.

5. Attaching Polar Polymers to Silica Surfaces

Preparation of polar polymer covalently bonded to silica surfaces in this thesis is done by *grafting*, and these reactions can be divided into two major approaches, that are both widely used. A *grafting from* process proceeds via conversion of the
grafting substrate into a macro-initiator, by introduction of a surface functional group capable of initiating the chain propagation of a monomer, whereas a ‘grafting to’ scheme proceeds via immobilization of short, terminal-functionalized polymers (telechelic oligomers) onto the surface. The ‘grafting from’ approach is often favored over ‘grafting to’ in surface modification of solid substrates by polymers because it typically yields thicker grafted layers of higher graft density. Both conventional and “controlled/living” free radical polymerizations are used in this thesis.

5.1. “TRIS-WAX” Silica [Paper I]

5.1.1. Atom Transfer Radical Polymerization (ATRP)

ATRP was developed by the Polish chemist Krzysztof Matyjaszewski in 1995. Among other “controlled/living” radical polymerizations (CRP), e.g., nitroxide-mediated polymerization (NMP), reversible addition-fragmentation chain transfer polymerization (RAFT), the ATRP method relies on intermittent formation of an active propagating species.

The generalized mechanism of an ATRP reaction is outlined in Scheme 2. An ATRP system requires reversible activation of a “dormant” species (such as an alkyl halide) and an ATRP catalyst consisting of a transition metal halide and a complexing ligand (regularly of polyamine type) to activate the dormant species. The activation takes place via homolytic cleavage of the alkyl-halogen bond R–X by transfer of the halogen atom X from R–X to a transition metal complex (Mt/L). This generates an organic radical R’ which can then propagate (at a rate constant k_{act}) with vinyl monomers (at rate constant k_p). The released halide is meanwhile attached as an additional ligand to the transition metal/ligand complex, Mt^{n+1}X/L, which metal assumes an oxidation state +1 higher due to the redox reaction with the halide. Essential for the ATRP mechanism is a reversible deactivation by back transfer of the atom X from the transition metal Mt^{n+1} X/L to the oligomer radical species, which reforms a halide-capped dormant polymer chain. The rate constant of this ‘capping’ reaction, k_{deact}, has to be significantly higher than k_{act} in order for a controlled polymerization to take place. While uncapped, radicals can propagate (k_p) but also terminate in a bimolecular reaction (k_t), but since k_{deact} \gg k_{act} the likelihood of this second order reaction is greatly diminished.
When properly set up, an ATRP polymerization can keep the growing terminals ‘alive’ long after the monomer has been consumed, which provides a possibility of formation of block copolymers, which in the design of stationary phase surfaces enables the formation of stratified grafts. Similar tricks can be pulled by other CRP techniques, but the advantage of ATRP over other CRP processes is a commercial availability of ATRP catalyst ‘kit’ components (alkyl halide, transition metals, and complexing ligands) and the wide range of monomers of vastly different polarity that can be polymerized (the most notable excepting being unprotected acids). The dynamic equilibrium between dormant species and propagating radicals can be adjusted for a given system by varying the complexing ligand of the catalyst\textsuperscript{105}. The rate of polymerization increases with initiator concentration, and activator to deactivator concentration ratio. One of the more significant advantages of ATRP in the preparation of surfaces for HILIC is finally that the technique can be run in fully or partly aqueous solutions.

\[ \text{Scheme 2. ATRP mechanism.} \]

5.1.2. TRIS Functionalized Silica Particles

Tris(hydroxymethyl)aminomethane (in short TRIS) is a small, highly hydrophilic compound that has been widely used as buffer compound in biochemistry. TRIS has also found applications in chemical modification of macroporous materials on the basis of 2,3-epoxypropyl methacrylate (glycidyl methacrylate; GMA), for use as chelating sorbent for boron compounds in aqueous samples\textsuperscript{106-108}, as ligand on soft gels for separation of biomacromolecules\textsuperscript{109}, and on porous silica for use as affinity media for lysozyme\textsuperscript{110}.

In Paper I, TRIS bonded to silica through an imino linkage C–NH–C is synthesized via a three-step reaction as shown in Scheme 3.
The porous silica particles used as a starting substrate are first modified with 3-bromopropyl trichlorosilane to form an ATRP macroinitiator and then grafted with glycidyl methacrylate (GMA) by controlled atom transfer radical polymerization (ATRP) in order to introduce an oxirane-carrying reactive tentacle layer on the silica surface. The grafted material is thereafter subject to an oxirane ring opening reaction with TRIS to yield a polymer-bound equivalent of the highly hydrophilic TRIS functionalities, namely Si-pGMA-TrisA (in Paper III and later in the thesis referred to as TRIS-WAX). The choice of a primary alkyl bromide (3-bromopropyl) as an ATRP initiator over more customary secondary or tertiary alkyl bromides was made on purpose due to its higher expected activation energy. This compensated for hydrolysis and other moisture related problem that are likely to occur on a silica surface, and provided a longer storage time for the ATRP macroinitiators. The exact ATRP reaction conditions are shown in the footnote of Table 1, Paper I. The molar ratios of ATRP reagents to silica macroinitiator and GMA monomer was [CuBr]:[CuBr₂]:[PMDTA]:[Si-Br]:[GMA]= 1:0.25:1.3:1:100, leading to successful incorporation of GMA monomer onto the silica particles, as confirmed by IR spectroscopy. The final synthesis step was an exhaustive acidic hydrolysis of non-reacted oxirane groups on the Si-pGMA-TrisA particles, which was done under acetic catalyzed conditions following the procedure reported by Shaw et al.\textsuperscript{111} for GMA bulk polymer.
The successful and exhaustive hydrolysis of the oxirane groups of GMA monomer by an aqueous acetic solution of pH 3.0 in 24 h at 60 °C was confirmed by $^1$H NMR chemical shifts (see Figure 3, Paper I). Therefore this hydrolysis procedure was used on the Si-pGMA-TrisA particles to form the final material, namely hydrolyzed Si-pGMA-TrisA or TRIS-WAX. Satisfied functionalities obtained on the TRIS-WAX are proven by different chemical characterization techniques including diffuse reflectance FT-IR, X-ray photoelectron spectroscopy (XPS), and elemental analysis. Additional characterization of the surface charge of the obtained TRIS-WAX material is accessed by $\zeta$-potential measurements that are shown in Paper II. Changes in physical properties like porosity and surface area of the TRIS-WAX particles upon functionalization and polymerization were determined by nitrogen cryosorption according to the Brunauer-Emmett-Teller (BET) principle (section 6.1.1). Chromatographic application of the TRIS-WAX material for separations of nucleic bases, small organic acids, and common nucleotides under mixed HILIC and weak anion exchange (WAX) conditions shows good results and a considerably higher retentivity than the unmodified silica.

5.2. “TRIS-Amide” Silica [Paper II]

5.2.1. Conventional free radical polymerization

Free radical polymerization (FRP) is one of the most versatile polymerization schemes available due to its simple and relatively non-specific nature.112 Another advantage of the chain growth mechanism is the facile reactions it provides for the polymeric free radical terminals with other chemicals that may be added to control for instance chain length and branching. In FRP a polymer forms by the successive incorporation of monomer entities by the free radical at the polymer terminal. The growing macro-radical is initially formed by an attack onto a monomer molecule by a free radical, derived by thermal or photochemical decomposition of unstable molecules called initiators (although other modes of initiation such as redox and ionization also exist). The polymerization thereafter proceeds by chain reaction, whereby new monomer molecules are bonded to the terminal by one of the electrons of its unsaturated bond. The other electron of the pair forming the unsaturated bond then propagates to form a new radical terminal. The addition of non-radical molecules containing for instance thiol groups to the free radical ends quenches the growing chain and results in termination. Finally two propagating chain terminals may combine to form a sigma bond, or they may disproportionate to form one saturated and one
unsaturated terminal, where the latter constitutes a macro-monomer that can be incorporated in another growing chain as a branch.

In Paper II, we chose to attach a thermally decomposable peroxy-type initiator onto the silica surface to form a macro-initiator for polymerizing the amphiphilic monomer \( N-[\text{tris(hydroxymethyl)methyl}]\text{acrylamide} \) (TRIS Acrylamide) by a free radical polymerization thermally initiated process. The general structure of a peroxy-type initiator is given in Figure 25.

\[
R1-O-O-R2
\]

Figure 25. Structure of peroxy-type initiator.

An important property of a thermal initiator is its half-life, \( t_{1/2} \), at a certain temperature, given by Equation 7,

\[
t_{1/2} = \frac{\ln 2}{k_d}
\]

where \( k_d \) is initiator decomposition rate in a given solvent. The half-life is the time period during which half of the initiators initially present are decomposed, and in practice, the choice of an initiator suitable for a specific polymerization is characterized by the temperature at which its half-life is 10 hours. These temperatures range between 20 to 120 °C, depending on the initiator structure. Extensive data on decomposition rates and activation parameters of initiators can be found in the Polymer Handbook by Brandrup et al.\textsuperscript{113} and also in trade literature available from commercial suppliers, for example on the Sigma-Aldrich website\textsuperscript{114}.

5.2.2. Grafting of TRIS-acrylamide from peroxidated Silica [Paper II]

In Paper II, a neutral hydrogel material analog to TRIS-WAX, namely TRIS-Amide was prepared. What makes the TRIS-Amide different from TRIS-WAX is that the TRIS functionalities in TRIS-Amide are linked to the graft chains by an amide bond instead of through a C–N–C–imino linkage. Preparation of the TRIS-Amide material was actually more straightforward than the TRIS-WAX, since a proper monomer, \( N-[\text{tris(hydroxymethyl)methyl}]\text{acrylamide} \) was readily available. Stationary phase synthesis based on TRIS Acrylamide was in fact introduced already thirty years ago by Boschetti, who published a procedure for the synthesis of ‘Trisacryl’-polymer particles for medium-pressure ion-exchange chromatography of proteins and other biomolecules\textsuperscript{115}. Yet until very recently the use of TRIS Acrylamide modified silica has not been tested in HILIC. A couple of
months ago Peng et al. published a work where they had synthesized a TRIS Acrylamide-grafted silica phase from a surface-bound azo-initiator and tested it for HILIC separation of nucleoside and peptides\textsuperscript{16}. The authors concluded, based on chromatography of a variety of test solutes, that the phase expressed both hydrophilic interaction and a WAX type ion exchange retention pattern, where the latter attributed to residual amino groups originating from the aminopropylated silica that had been used as a starting point in the synthesis. In Paper II, we had instead chosen unmodified silica as substrate to synthesize an amide-bonded TRIS stationary phase without an underlying WAX amino functionality and combined this with a “grafting from” procedure using tert-butylperoxy functionalized silica to trigger a thermally initiated free radical polymerization. The detailed synthesis route for the TRIS-Amide material (or Si-pAcrylamide-TRIS) in Paper II is shown in Scheme 4.

![Scheme 4. Schematic diagram showing a) chlorination of silica surface with thionyl chloride, b) attachment of tert-butyl peroxide groups onto chlorinated silica, c) the process of surface graft polymerization of N-[Tris(hydroxymethyl)methyl] acrylamide from the tert-butyl peroxy functionalized silica surface. [Paper II].](image)

The use of tert-butyl peroxide attached to silica as a tethered initiator for radical polymerization was first reported by Tsubokawa and Ishida\textsuperscript{17} and has successfully been used to polymerize highly hydrophilic monomers, containing both zwitterionic\textsuperscript{58,59} and non-ionic polyhydroxy groups\textsuperscript{40}. The TRIS-Amide material synthesized by this procedure has a substantial grafting yield of 13.8 % total C by
elemental analysis, which corresponds to 29\% organic graft on the silica at polymerization temperature and time of 80 °C and 18 hours, respectively. The use of partly aqueous ethanol/water mixture rich in organic modifier content (85:15 \%, v/v) as polymerization solvent helped keeping the amphiphilic monomer dissolved and facilitated efficient graft polymerization from the hydrophobic tert-butylperoxide activated silica surfaces, which is otherwise difficult to accomplish with an amphiphilic vinylic monomer\(^{40}\). The TRIS-Amide material was further characterized by different spectroscopy methods, and the changes in surface area and mesopore distribution as a result of the surface modification reactions were determined by nitrogen cryosorption. In terms of chromatographic characterization under HILIC conditions, the neutral amide-bonded TRIS stationary phase features good separation efficiency, a comparatively high retention, and a selectivity that differs from the imino-bonded TRIS-WAX phase and most commercially available HILIC phases (Papers II-III).

5.3. **Stratified Bis-acrylamide/MPC Silica [Paper IV]**

5.3.1. **Iniferter-mediated polymerization**

Since the discovery of living polymers by Szwarc\(^{118}\) in 1956, several ‘living’ radical polymerization schemes have been established, allowing syntheses of well-defined polymers/block copolymers of desired structure\(^{119}\). In a living radical polymerization, the ‘living’ radical exists as a dormant species that can induce further radical polymerization. The dormant species may be activated spontaneously thermally, or by the presence of light at a suitable wavelength. Long before the discovery of what are now known as the core living radical polymerization schemes (including NMP\(^{120,121}\), ATRP\(^{122,123}\), and RAFT\(^{124,125}\)), Otsu et al.\(^{126,127}\) published in 1982 a way of controlling photopolymerizations by means of an ‘iniferter’. The term refers to different compounds bearing dithiocarbamyl groups\(^{128,129}\), or carbon-carbon\(^{130,131}\) or azo\(^{126,132}\) bonds as dormant species, and is a combination of *initiator* transfer-agent *terminitor*\(^{126,133}\). Iniferters are thus capable of both inducing and controlling living radical polymerizations that lead to various functional, block, graft, star, and crosslinked polymers\(^{133}\). The discovery of iniferters was made by Otsu’s group when they reported a living like radical polymerization of styrene and methyl methacrylate in solution with new types of photopolymerization initiators based on dithiocarbamate derivatives\(^{134,135}\). These compounds were in fact photoiniferters, where both the initiation and the reversible termination are triggered by switching on and off a light source with
wavelength capable of breaking the bond responsible for the reversible capping. The progress of the polymerization results in fragments of the iniferter compound being incorporated into either end of the final polymer chain. If the goal is to prepare a telechelic oligomer, the propagating group can often be traded in for a desirable end group by selective chain transfer.

In Paper IV, we chose to incorporate a \( N,N \)-diethyldithiocarbamate (DEDT) salt onto 3-bromopropylated silica to form a 3-DEDT-propyl functionality that is able to act as photoiniferter for the surface block-photograft copolymerization of \( N,N' \)-methylenecisacrylamide (BIS), followed by 2-methacryloyloxyethyl phosphorylcholine (MPC) onto the pore surfaces. Photoiniferter mediated polymerization is a controlled polymerization scheme based on the photochemistry of dithiocarbamates under UV irradiation, as presented in Scheme 5.

![Scheme 5. Proposed scheme of a sequential surface-initiated "controlled/living" photoiniferter-mediated graft polymerization of monomers M followed by N from silica, leading to stratified layers, composed of poly(N) on top of poly(M). Note that the DC fragment from the iniferter is present in the terminals (i.e., distally on the surface) after the system as stopped reacting.](image)

In this process, polymerization of a monomer M with a photoiniferter R-DC proceeds first via a photolytic dissociation of the R-DC molecule (the initiation step) into the radicals R• (a radical that is capable of initiating a radical polymerization) and DC• (a dithiocarbamyl radical lacking initiation capability). The role of the dithiocarbamyl radical DC• is to recombine with the growing radical R• to form a bond of relatively low binding energy, which is homolytically photocleavable by light of proper wavelength. In a photoiniferter, both the initiation and the reversible termination are thus triggered by light. In the case of the \( N,N \)-diethyldithiocarbamate (DEDT) group covalently bonded to the propylated silica surface via the carbon-sulfur bond, irradiation by UV light causes formation of a propylated silica radical (initiator), which is very reactive towards vinylic monomers and also towards the \( N,N \)-diethyldithiocarbamyl radical (scavenger). Although the \( N,N \)-diethyldithiocarbamyl radical is non-reactive for
initiation, it is highly reactive for primary radical termination. It thus recombines with the propagating oligomer radical to form a dormant species. As the polymerization proceeds, the DC part of the iniferter consequently propagates as the terminal of the grafted polymer chain. When all monomer is consumed or the radiation is terminated, this dormant species will remain in the terminal and can be used to induce further radical polymerization if a new monomer N is added and the radiation is continued. Photoiniferted polymerization is therefore ideally suited to form block graft-copolymerized surfaces. The only drawback is the need to reach the treated surface by UV irradiation. The technique is therefore suited only for thin layers, or strongly agitated suspensions with reasonably low absorption of the wavelength being used to promote dissociation of the R–DC bond.

The main advantage of using a photo-initiator in polymerization is that we can define exact start- and endpoints of the polymerization by means of the duration of the irradiation. In addition, the rate of (most) photo-initiator decomposition is almost independent of the reaction temperature, but depends strongly on the (UV) light wavelength and intensity. This method allowed sequential grafting of various monomers onto the silica surface due to the "living" properties of the end-capped DEDT groups in the propagating polymer side-chain end via the surface photoiniferter-mediated graft copolymerization.133

5.3.2. Synthesis of silica with MPC overlaid on bis(acrylamide)

The pathway for the modification of the silica surface with diethylammonium N,N-diethylthiodithiocarbamate to form a 3-N,N-diethylthiodithiocarbamate propyl covalently bonded silica iniferter via a two-step reaction is outlined in Scheme 6, steps a+b. Silica was first functionalized by the reaction with surface silanol groups with 3-bromopropyl trimethoxysilane. Alkoxy)silane groups are well-known for reacting readily with silanols. A 3-bromopropyl silane agent was chosen instead of other widely used haloalkylsilyl agents such as 3-chloropropyl trimethoxysilane136,137,138 since the bromide is a better leaving group than the chloride when the haloalkyl groups is intended to react with the N,N-diethylthiodithiocarbamate ion via a nucleophilic SN₂-substitution with high yield. Both 3-bromopropyl trimethoxysilane and 3-chloropropyl trimethoxysilane were tested in this reaction and XPS analysis showed that a higher amount of N,N-diethylthiodithiocarbamate groups was obtained on the silica surface with the 3-bromopropyl activated silica. The reaction of silica with 3-(bromopropyl) trimethoxysilane to form Si-Br particles (Scheme 6, step a) was followed by
reaction with diethylammonium $N,N$-diethyldithiocarbamate to produce the Si-DEDT macriniferter (Scheme 6, step b). Also this reaction yielded the desired functionalization, as verified by IR, XPS and elemental analysis of the Si-Br and Si-DEDT surfaces. In HPLC stationary phase synthesis, Cheong et al. has demonstrated the use of DEDT functionalized phase silica for grafting of polystyrene for use in reversed-phase chromatography$^{136,137}$, and the versatility of silica-immobilized DEDT as starting point for both thermally and photo-iniferter polymerization has been demonstrated by others with a variety of monomers$^{138-142}$.

![Scheme 6](image)

**Scheme 6.** Schematic diagram showing a) bromination of silica surface with 3-bromopropyltrimethoxysilane, b) attachment of $N,N$-diethyldithiocarbamate groups onto brominated silica, c) the process of surface photoiniferter-mediated polymerization of BIS from the $N,N$-diethyldithiocarbamate functionalized silica surface, d) the process of surface photoiniferter-mediated polymerization of MPC from the $N,N'$-methylenebisacrylamide grafted silica surface.

**Paper IV**

In **Paper IV**, successful introduction of DEDT groups onto the silica surfaces via the two-step reaction allowed us to use it for sequentially grafting of BIS and MPC monomers through a photochemically triggered iniferter graft copolymerization (Scheme 6, steps c and d). The intermediate silica grafted with BIS (Si-BisA) only as well as the final diblock graft copolymerized silica with MPC (Si-BisA-pMPC) on top of BIS (Si-BisA), were both characterized by an array of spectroscopic and chemical methods as in Papers I and II. The results confirmed that the intended block graft had taken place, and that the graft density was within the same range as the previously prepared TRIS-WAX and TRIS-Amide materials; in other words the phase loading appeared to be suitable for use as stationary phases in HILIC.

Chromatographic evaluation in HILIC mode was carried out using a set of fourteen different compounds, including neutral nucleic bases and amino acids, positively charged amines, and negatively charged benzoic acids. The retention pattern for
these solutes showed that the diblock graft material (Si-BisA-pMPC) was suitable for use as a HILIC stationary phase, featuring higher retention compared to bare silica and the intermediate Si-BisA materials, and possessing a selectivity that is different from the commercially available zwitterionic phosphorylcholine type material ZIC-cHILIC.

6. Physical and Chemical Characterizations

Separation materials for HPLC are critically dependent both on their physical constitution and the chemical properties of the interfacial layers. Some of these parameters can be assessed by direct measurements, whereas others have to be determined in more indirect ways. Below follows a summary of the physical and chemical characterization techniques that have been used to characterize the materials produced as a result of the work accounted for in this thesis.

6.1. Physical Characterization Techniques

Among the more critical characteristics of stationary phases for HPLC is the pore size distribution, which affects both the maximum size of solutes that can be separated on a particular phase, and also the phase ratio and thereby the retention through the inverse relationship between pore diameter and surface area. Pores in silica material are in the micro- and mesopore range, and a full characterization of grafted materials will reveal a) if the grafting has been successful (the pore volume should decrease); b) provide an estimate of the grafting thickness (from the decrease in average pore diameter). A careful surface area and pore size analysis can also telltale if the grafting has caused the formation of bottleneck pores, which can cause slow mass transfer by limiting diffusion into and out of fully porous materials.

6.1.1. Cryosorption according to Brunauer, Emmett, and Teller (BET)

The adsorption of gases on solids at cryogenic temperature for the probe gas has been used for three quarters of a century to measure specific surface areas and to determine the porosity of a wide variety of materials. This method is based on theory developed by Brunauer, Emmett, and Teller\textsuperscript{143}(BET) in 1938 when they extended the sorption isotherm theory developed as a result of a visionary work by Langmuir\textsuperscript{144} in 1916 from mono- to multi-layer adsorption, with a specific aim of accurately determining the specific surface area of solids. In modern automated
cryosorption instruments, the sample under test is first evacuated. Exact aliquots of gas are thereafter added and an incremental pressure:volume curve is recorded until the pressure approaches atmospheric. The added gas is thereafter pumped out in metered quantities to establish isotherms describing the coverage of gas in the pore space of the tested material at advancing and receding surface coverage.

With these pressure-dependent sorption data, calculations can be extended from surface area to pore size distribution and pore volume via the “BJH method”, due to Barrett, Joyner and Halenda. In the multipoint nitrogen gas sorptiometry, the adsorption of nitrogen engenders the sum of non-specific and specific molecular interactions if the absorbent in question is hydroxylated porous silica. Supports for stationary phases are considered to be composed of micropores (diameter, $d < 2 \text{ nm}$), mesopores ($2 < d < 50 \text{ nm}$), macropores ($d > 50 \text{ nm}$), or they may be non-porous.

Reproducibility of the experiment depends on establishing a known surface state of the silica, which is controlled by thermal treatment (outgassing). This method (outgassing followed by measurements) is good for determining specific surface area of nonporous or mesoporous silica gels. For silica gels with extensive microporesystems, the results are more difficult to interpret.

In Papers I, II and IV, BET measurements were used to access the changes in surface area and porosity of the silica particles after polymerization. Mesoporous silica substrate (Kromasil, Bohus, Sweden) used in this thesis has average surface area, pore volume, and pore diameter of $190 \text{ m}^2/\text{g}$, $0.83 \text{ cm}^3/\text{g}$ and $13.8 \text{ nm}$, respectively. In general, graft polymerization of silica particles with a monomer will partly fill up the pore space with polymer tentacles which tend to reduce both the volume and diameter of the pores as well as the specific surface area of the grafted material in relation to the silica substrate used. Besides, in “controlled/living” radical polymerization schemes such as ATRP and iniferter-mediated polymerizations, the grafting yield typically will be lower as a consequence of slower rates of monomer incorporation compared to conventional free radical polymerization. This shows up in the cryosorption measurements as lower reductions in the BET surface area and BJH total pore volume, and is also reflected in the pore size distribution of grafted silica particles synthesized by the “controlled/living” radical polymerizations when compared to conventional free radical polymerization.

As expected, the TRIS-WAX (Paper I) and Si-BisA and Si-BisA-pMPC (Paper IV) materials that have been synthesized by ATRP and iniferter-mediated photopoly-
merization had the surface areas, pore volumes slightly lower than those of bare silica; 166 m²/g surface area and 0.76 cm³/g pore volume for TRIS-WAX (Paper I, Table 2), 181 m²/g surface area and 0.61 cm³/g pore volume for Si-BisA and 146 m²/g surface area and 0.44 cm³/g pore volume for Si-BisA-pMPC (Paper IV, Table 3). In Paper II, where the TRIS-Amide material was synthesized by conventional free radical polymerization, the BET surface area was substantially reduced to 103 m²/g and the pore volume was as low as 0.38 cm³/g (Paper II, Table 4). The pore diameters of all grafted silica were also reduced after polymerizations (see Paper I, Table 2; Paper II, Table 4; and Paper IV, Table 3). By the cryosorption measurements, we could hence confirm successful grafting of a variety of different monomers onto mesoporous silica particles by the impact that polymer layers have on the material surface and the pore space.

6.2. Chemical characterization Techniques

In order to identify and quantify chemical functional groups on the silica surface and hence the successful chemical functionalization of the silica surfaces, various chemical characterization techniques were employed to access the chemical properties (e.g. functional groups, elemental composition and concentration, surface charge) of the silica surface. Understanding chemical properties of the silica particles is of importance since the solute retention as well as chemical interactions in HPLC in general and specifically in HILIC take place at the silica particle-eluent (i.e. water/organic solvent mixture) interface.

Chemical characterization techniques are often classified into bulk and surface analysis techniques, depending on whether the techniques provide bulk or surface chemistry of the particles, which is in function of sample analysis depth. In general, bulk techniques e.g. infrared spectroscopy (IR), elemental analysis (EA) can provide information on chemical functional groups, and chemical composition of the top layer (in µm thickness) of the specimen, while the surface techniques give information on chemical composition and concentration and surface charge of the top layer in nm thickness e.g. X-ray photoelectron spectroscopy (XPS), electrophoretic light scattering (ELS).
6.2.1. Bulk techniques

6.2.1.1. Diffuse Reflectance Infrared Fourier Transform spectroscopy (DRIFTS)

Fourier transform infrared spectroscopy (FT-IR) is a technique which is used to obtain an infrared absorption or emission spectra of a solid, liquid or gas\textsuperscript{146}. It is generally not possible to identify an unknown compound only by IR spectroscopy. Nevertheless, it is quick and relatively inexpensive and very good for identifying functional groups, and hence preliminary information on the identity or structure of a molecule. An IR spectrum example plotting wave number (cm\textsuperscript{-1}) against IR absorbance is shown in Figure 26, recorded in the often used mid-infrared range (4000–400 cm\textsuperscript{-1}).

![Figure 26. Superimposed IR spectra of a) unmodified silica; b) bromopropyl functionalized silica; and c) GMA grafted silica. Note the peaks attributable to silanol groups at 3200-3700 cm\textsuperscript{-1} in (a), the aliphatic alkyl chain at \( \sim 2950 \text{ cm}^{-1} \) in (b) and a carbonyl peak at 1732 cm\textsuperscript{-1} in (c). [Paper I] ](image)

Instrumentation and sample preparation techniques for transmission and reflection IR techniques such as attenuated total reflection (ATR), and IR microscopy can be found elsewhere\textsuperscript{147}. In Papers I, II and IV, FT-IR in diffuse reflection mode was used to obtain IR spectra from the rigid solid silica samples. This technique is well suited for solids and powders with preferred particle size smaller than 10 \( \mu \text{m} \) (\textit{i.e.},...
not exceeding the wavelength of the incident radiation). Spectra of powders and rough surfaces can be recorded by illuminating these surfaces with IR radiation and collecting sufficient scattered radiation with ellipsoids and paraboloids as presented in Figure 27. This technique is a fast measurement of powder samples and provides information from the bulk matrix.

![Instrumentation and mechanisms generating infrared spectrum of a powder. Adapted from ref.148.](image)

In Papers I, II, and IV, diffuse reflectance FT-IR spectra were acquired with a Bruker (Ettlingen, FRG) Equinox 55 FT-IR instrument and the bands used to identify different functional groups were 3200-3700 cm⁻¹ for hydroxyls from silanols and tris(hydroxymethyl) moieties of the polymer grafts; 3320-3520 cm⁻¹ for the secondary amine–NH– in TRIS; 2850-2990 cm⁻¹ for aliphatic alkyl groups–CH₂–; 1725-1732 cm⁻¹ for the ester group –(C=O)–O–, and the amide group –(C=O)–NH– appearing at two peaks at 1650-1662 cm⁻¹ and 1525-1550 cm⁻¹ (see IR spectra of the functionalized and grafted silica particles in Paper I, Figure 2; Paper II, Figure 2 and Paper IV, Figure 3).

### 6.2.1.2. Elemental Analysis

This destructive combustion method gives a total (bulk) elemental composition of the material being analyzed. Since the silica substrate lacks elements other than silicon, oxygen, and hydrogen, combustive elemental analysis can provide exact stoichiometric ratios between carbon and hetero atoms (nitrogen, phosphorus, and sulfur) in the grafted layers of materials being characterized. A CHN (carbon, hydrogen, and nitrogen), S (sulfur) and P (phosphor) combustion type analyzer was consequently used to determine the total amount of specific elements in the functionalized and grafted silica materials.
In **Paper I**, a CHN elemental analysis experiment was performed on the TRIS-WAX material, giving a total carbon content of 5.5 % corresponding to 11.0 % organic polymer on the stationary phase, with a total amount of nitrogen of 0.56 %. The molar ratio of N:C determined by bulk elemental analysis is thus 1:11.4, which is close to the theoretical molar ratio 1:11, calculated from the monomer unit structure, confirming the successful functionalization and grafting.

In **Paper II**, a similar CHN experiment was done on the TRIS-Amide particles. A total carbon content of 13.8 % corresponding to 29 % organic polymer grafted on the silica particles with a total amount of nitrogen of 2.2 % were found. The molar ratio of N:C determined by bulk elemental analysis is thus 1:7.3, which is again close to the theoretical molar ratio of 1:7, as calculated from the monomer unit structure. The high total amount of the polymer grafts on silica surfaces observed by the elemental analysis on the TRIS-Amide particles was also consistent with the substantial decreases in surface area and pore volume in the grafted material, measured by BET as discussed above.

In **Paper IV**, combustive elemental analyses including CHN, sulfur and phosphorous were carried out on 3-bromopropylated silica (Si-Br), DEDT functionalized silica iniferter (Si-DEDT), and silica particles grafted with BIS monomer (Si-BisA), and subsequently grafted with MPC monomer (Si-BisA-pMPC). The results shown in **Paper IV**, Table 2 confirmed the successful functionalization and grafting. Only carbon element with a total percentage of 2.0 % was found in the Si-Br particles. This amount was doubled to 4% in the Si-DEDT particles, where nitrogen and sulfur amounting to 0.5 % and 2.1%, respectively, were also found. This could be attributed to the dithiocarbamate group of the DEDT moiety. A further increased total carbon content of 6.7 % was found in the silica particles grafted with BIS (Si-BisA). This corresponds to 12 % of organic polymer on the stationary phase. The total amount of nitrogen concurrently increased to 1.5 %, attributed to the amide groups from the BIS graft chains. The total amount of sulfur found in the Si-BisA particles was 2.0 %, verifying that the DEDT groups remained on the polymer in their role as capping agents for the dormant polymer chain. Overall, these results confirmed the successful grafting of BIS onto silica surfaces by 3-DEDT propylated silica iniferter under a UV irradiation time of four hours and also that the DEDT groups incorporated into the polymeric chain ends were intact and stable during the photopolymerization and the washing procedure. Thus, the Si-BisA particles were further successful used for the second stage grafting with MPC monomer, the success of which was again confirmed by elemental analysis of the graft co-
polymerized silica (Si-BisA-pMPC) where the total amount of carbon and nitrogen had increased to 11.2 and 1.8 %, respectively. Along with other characterization steps and chromatographic evaluations, this serves to prove that the sequential photografting scheme based on a DEDT macroinitiator on the silica surfaces was indeed a feasible route to stationary phases with stratified graft layers. A total amount of 1.2 % phosphor was also found in the Si-BisA-pMPC particles, which is attributed to the phosphorylcholine functionality of the polymeric MPC top graft.

6.2.2. Surface techniques

6.2.2.1. X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS), previously known as electron spectroscopy for chemical analysis (ESCA), is a spectroscopic technique that can be used to obtain information on the chemical and electronic states of elements on the surface of materials, in addition to providing quantitative elemental composition. XPS spectra are obtained by irradiating the surface under test with a beam of monochromatic X-rays and monitoring the flux and kinetic energy of the photoelectrons emitted from the upper 1 to 10 nm of the material being analyzed.

The process leading to the emission of photoelectrons is illustrated in Figure 28. The surface to be analyzed is first placed in a vacuum environment and then irradiated with photons in the X-ray energy range. The surface atoms emit electrons (photoelectrons) after direct transfer of energy from the photon to the core-level electrons. These emitted electrons are characterized by binding energy (BE) and counted number. The energy of a photoelectron is related to the atomic and molecular environment from which it originated, and the number of electrons emitted is proportional to the concentration of the emitting atom in the sample.
Figure 28. Photoemission of an oxygen atom in a XPS experiment[149].

XPS analysis of a surface will provide approximate surface (2-10 atomic layers, or 0.5-3 nm thickness) elemental composition (qualitative) and amount (quantitative) information on all elements present, except H and He. Unlike bulk elemental analysis experiments, XPS analysis also gives information about the molecular environment (oxidation state, bonding atoms, etc.) of the top layers. Therefore, XPS analysis enhances specification of the chemical structure of a surface.

Examples of XPS spectra are shown in Paper IV, Figure 3. Through Papers I-II, and IV, XPS analyses have been determined on the washed and dried material after each reaction step. All binding energies (BE’s) are referenced to the C 1s C-H peak at 285.0 eV. Other binding energies characteristic of different functional groups obtained by XPS analyses of the functionalized and grafted silica surfaces in Papers I-II and IV are summarized in Table 1.

Table 1. Characterization of functionalized and grafted silica particles with XPS.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Bond energy (eV)</th>
<th>Peak</th>
<th>Bond energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 1s [C–(C,H)]</td>
<td>285</td>
<td>C–Br 3d 5/2; C–Br 3d 3/2</td>
<td>70.31; 71.36</td>
</tr>
<tr>
<td>C 1s [C–(O,N,S)]</td>
<td>286.5</td>
<td>S–C 2p 3/2; S–C 2p 1/2</td>
<td>162.05; 163.22</td>
</tr>
<tr>
<td>C 1s [O=C–N]</td>
<td>288.1</td>
<td>S=C 2p 3/2; S=C 2p 1/2</td>
<td>163.62; 164.8</td>
</tr>
<tr>
<td>C 1s [COO]</td>
<td>289</td>
<td>PO2(OR)2 2p 3/2; PO2(OR)2 2p 1/2</td>
<td>133.0; 133.8</td>
</tr>
<tr>
<td>N 1s [N–(C,H)]</td>
<td>399.2-400</td>
<td>PO2H(OR)2 2p 3/2; PO2H(OR)2 2p 1/2</td>
<td>134.2; 135.0</td>
</tr>
<tr>
<td>N 1s [N’–(C,H)]</td>
<td>402.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The obtained XPS data including characteristic binding energy to each atom and its atomic concentration gave information on the surface stoichiometric atomic composition that were in good agreement with the expected ratio calculated from
the chemical structure of the functionalized and grafted silica surfaces, which confirmed the successful functionalization and grafting (Paper I, Table 1; Paper II, Table 2 and Paper IV, Table 1).

6.2.2.2. Electrophoretic Light Scattering (ELS)

ELS, also known as laser Doppler electrophoresis (LDE) or laser Doppler velocimetry (LDV), is used for \( \zeta \)- (zeta)-potential measurement\(^{150}\). The \( \zeta \)-potential is an important surface property of particles that reflects their surface charge, probed in the "slipping plane". When a charged particle is suspended in a liquid buffer solution, it is surrounded by an electrical double layer, the thickness of which is dependent on the surface charge density and the concentration and composition of the electrolyte solution. Depending on their surface charge, suspended particles may remain as singlets or form agglomerates/aggregates of the charge density that is insufficient to prevent collisions by electrostatic repulsion. A cartoon showing the location of the \( \zeta \)-potential in the electrolyte shell surrounding a charged particle is presented in Figure 29.

![Figure 29. Representation of \( \zeta \)-potential\(^{151}\).](image1)

In this figure, the liquid layer surrounding the particle (the electrical double layer) exists as two parts; an inner region (the Stern layer) where the ions are strongly bound and an outer (diffuse) region where they are less firmly associated. Within the diffuse layer there is a notional boundary called "slipping plane", inside which the ions and particles form a stable environment. If a charged particle is subject to tangential stress, such as the viscous drag acting as counter-force in electrophore-

![Figure 30. Model of the electric double layer on a stationary bonded-phase surface. Reprinted with permission from Wiley-VCH \cite{153}.](image2)
tic \( \zeta \)-potential measurement, the liquid inside the slipping plane will move together with the particle. Since the velocity determined by a particle electrophoretic experiment is established by the balance of the driving force from the electric field and the opposed frictional force, the measured \( \zeta \)-potential will be the potential at the slipping plane. The \( \zeta \)-potential is therefore not an exact representation of the surface potential (surface charge), but the potential of practical interest in dispersion stability because it determines the interparticle forces\(^{152}\). A model of the electric double layer on the silica-bonded phase surface is proposed by Dziubakiewicz \textit{et al.}\(^{153}\) (Figure 30).

When silica particles are coated by a polymer, suspension of these particles in a buffer solution will form a dispersion, which stability depends on surface charge density (in turn dependent on surface ionization and ion adsorption), and on the \( \text{pH} \) and ionic strength of the buffer solution. Therefore, small changes in the \( \text{pH} \) or concentration ion can lead to drastic change in the \( \zeta \)-potential. Since the surface charge is expected to contribute significantly to the mixed hydrophilic/electrostatic interaction mechanism in HILIC, we measured the \( \zeta \)-potentials of the synthesized phases TRIS-WAX, TRIS-Amide, and Si-BisA-pMPC materials alongside their substrate Kromasil silica in different solutions, all designed to mirror the chromatographic elution conditions under which the materials have been evaluated. Results from such experiments are found, for example, in \textbf{Paper II}, Table 6; \textbf{Paper III}, Table 3; and \textbf{Paper IV}; Table 4.

In addition, four other commercially available HILIC stationary phases were also subject to \( \zeta \)-potential measurements in order to be able to compare the results from the imino- and amide bonded TRIS based phases and the zwitterionic phosphorylcholine type phase. These phases were selected to have markedly different chemical functional groups, namely sulfoalkylbetaine zwitterionic (ZIC-HILIC), anionic (Atlantis HILIC Silica), cationic (Purospher STAR NH\(_2\)), and neutral (Luna Diol). The \( \zeta \)-potential results of the TRIS-WAX, TRIS-Amide and the four commercial HILIC phases summarized from \textbf{Papers II and III} are presented in Table 2.
**Table 2.** ζ-potential measurements on the seven stationary phases [Papers II-III].

<table>
<thead>
<tr>
<th>Phase</th>
<th>Ammonium formate buffer, pH = 4.0</th>
<th>Ammonium acetate, pH = 6.8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mM</td>
<td>20 mM</td>
</tr>
<tr>
<td>Kromasil Silica</td>
<td>-14.6 ± 0.5</td>
<td>-12.9 ± 0.9</td>
</tr>
<tr>
<td>Atlantis HILIC Silica</td>
<td>-9.9 ± 0.1</td>
<td>-8.5 ± 0.1</td>
</tr>
<tr>
<td>TRIS-Amide</td>
<td>-9.1 ± 1.3</td>
<td>-4.8 ± 0.2</td>
</tr>
<tr>
<td>TRIS-WAX</td>
<td>14.5 ± 0.7</td>
<td>8.7 ± 0.7</td>
</tr>
<tr>
<td>PurospHER STAR NH₂</td>
<td>11.0 ± 0.2</td>
<td>5.2 ± 0.6</td>
</tr>
<tr>
<td>ZIC-HILIC</td>
<td>-13.8 ± 0.2</td>
<td>-8.6 ± 0.4</td>
</tr>
<tr>
<td>Luna Diol</td>
<td>-2.8 ± 0.2 a)</td>
<td>-2.1 ± 0.1 a)</td>
</tr>
</tbody>
</table>

ζ-potential values are reported in mV. Measurement conditions: Solvent, 80:20 % (v/v) acetonitrile/water with electrolytes added as indicated; Temperature, 25 °C. All ζ-potential values were recalculated in the Zetasizer software with a corrected viscosity of 0.4995 cPoise, a dielectric constant of 44, and a refractive index of 1.3452, at 25 °C for the solvent mixture used. a) Data from Paper III, measured in ammonium acetate buffer, pH 4.0.

It can be seen that the unmodified Kromasil silica has the highest negative surface charge among the tested materials, with a ζ-potential slightly less negative in both ammonium formate buffer pH 4.0 compared to ammonium acetate at pH ~6.8 and at the higher of the tested salt concentrations 20 mM, at both tested pHs. Negative and pH-dependent ζ-potentials are expected for neat silica due to dissociation of silanol groups, which is less favorable at low pH values. The less negative ζ-potentials at higher electrolyte concentration are rationalized by compression of the double layer. The negative ζ-potentials of Atlantis HILIC are slightly lower magnitude compared to those expressed by Kromasil silica, which is a not dedicatedly synthesized for HILIC. Also the supposedly neutral TRIS-Amide material expresses negative ζ-potentials under all the tested conditions and are lower (about half the magnitude) compared to those of the Kromasil silica substrate. This is due to some residual unreacted silanols on the grafted silica. On ZIC-HILIC, which carries a polymeric sulfobetaine layer, the ζ-potentials were negative and comparable to Kromasil silica, which is rationalized by the distal location of the sulfonic acid group in the sulfobetaine side chains [Paper II]. Among the stationary phases that expressed negative ζ-potentials, Luna Diol had the least negative ζ-potentials; more negative at the higher pH 6.8 at both concentration levels tested [Paper III]. The close-to-zero surface net charges of Luna Diol phase indicates either the least silanol activity of this stationary phase, or (more likely) that these residual silanol groups are better shielded by the neutral cross-linked polymeric diol layer compared to the tentacle layer as in TRIS-Amide material. For the TRIS-WAX material with secondary amine functionality, the measured ζ-potentials are positive and exceed the ζ-potentials shown by the PurospHER STAR NH₂ in ammonium formate.
buffer, pH 4.0 at both the concentration levels tested, and also in 5 mM ammonium acetate, pH 6.8. At 20 mM ammonium acetate, the \( \zeta \)-potential is slightly negative, most probably due to a preferential adsorption of acetate ions.

Table 3. Results from the \( \zeta \)-potential measurements on bare and grafted silica[Paper IV].

<table>
<thead>
<tr>
<th>Phase</th>
<th>Ammonium acetate, pH = 4.0</th>
<th>Ammonium acetate, pH = 6.8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mM</td>
<td>20 mM</td>
</tr>
<tr>
<td>Kromasil Silica</td>
<td>–15.6 ± 0.3</td>
<td>–12.9 ± 0.9</td>
</tr>
<tr>
<td>Si-BisA</td>
<td>–9.2 ± 0.4</td>
<td>–9.1 ± 0.2</td>
</tr>
<tr>
<td>Si-BisA-pMPC</td>
<td>–0.4 ± 0.1</td>
<td>–1.2 ± 0.2</td>
</tr>
</tbody>
</table>

Measurement conditions: Solvent, 80:20 % (v/v) acetonitrile/water with electrolytes added as indicated; Temperature, 25 °C. All \( \zeta \)-potential values were re-calculated in the Zetasizer software with a corrected viscosity of 0.4995 cPoise\(^{154}\), a dielectric constant of 44\(^{154}\), and a refractive index of 1.3452\(^{155}\), at 25 °C for the solvent mixture used.

In Paper IV, \( \zeta \)-potentials of Kromasil silica substrate, Si-BisA and the stratified Si-BisA-pMPC in different solutions are shown in Table 3. Kromasil silica expressed the most negative \( \zeta \)-potentials among the three phases. The supposedly neutral Si-BisA also showed negative \( \zeta \)-potentials with lower magnitudes compared to those expressed on the Kromasil silica, which is attributed to lower exposure of dissociated residual unreacted silanol groups, as discussed for the Luna Diol phase above. The \( \zeta \)-potentials of the Si-BisA varied with the same trend as for Kromasil silica under changing pH and salt concentrations. On the Si-BisA-pMPC material, which is composed of a tentacle zwitterionic phosphorylcholine layer on top of the cross-linked Si-BisA surface, the \( \zeta \)-potentials drastically decreased in absolute magnitude and were close to zero. The very low negative \( \zeta \)-potentials on the Si-BisA-pMPC material indicated the good surface coverage of the Si-BisA substrate with the tentacle MPC layer, and this tentacle layer capable of further shielding of the residual silanol groups. The \( \zeta \)-potential of the Si-BisA-pMPC varied only slightly with eluent pH, which is rational as the charge state of the zwitterionic phosphorylcholine is independent of pH within the tested range. The residual silanols appeared to be well shielded by the underlying Si-BisA layer, as mentioned above. At the higher electrolyte concentration 20 mM, \( \zeta \)-potentials became less negative at pH 6.8 which can be due to compression of the double charge layer, and more negative at pH 4.0, which is rationalized by the adsorption of acetate ions by the distal quaternary ammonium groups in the grafted phosphorylcholine side chains.
7. TRIS-WAX, TRIS-Amide and Stratified MPC Silica in HILIC

The intention of synthesizing the three stationary phases covered by this thesis was, of course, to create a new set of phases for HILIC mode separation, where the selectivity is complimentary to columns that already exist in the market. This can only be assessed by determination of retention times for a select set of solutes, chosen to probe the main interaction mechanisms expected to be seen in HILIC. As discussed in earlier sections, “true” HILIC is based on partitioning of solutes from the acetonitrile-rich eluent into a water-enriched layer established on the stationary phase surface by its polar groups. However, overlaid on this solvation-driven retention mechanism are also other, relatively strong interaction modes, such as oriented hydrogen bonding and electrostatic interactions\(^5\). The choice of solutes is therefore catchy, and we finally ended up with a probe set that incorporated nucleic bases, nucleotides, and organic acids. This chapter presents a collected account of the retention patterns observed with these probes on all phases synthesized in the work covered by this thesis.

7.1. Separation of Nucleic bases

In order to give a first, rough idea on how a polar packing will perform in HILIC separation condition, it is common to inject a characteristic test mixture composed of toluene as a void marker (\(t_v\)), and two polar nucleic bases uracil and cytosine to obtain fundamental chromatographic parameters such as retention factor (\(k'\)), efficiency (\(N\)), and selectivity (\(\alpha\)) that are used in the initial assessment.

In Papers I-II, and IV, the grafted silica phases TRIS-WAX, TRIS-Amide, Si-BisA and Si-BisA-pMPC particles have been packed into either a 50×2.1 mm i.d. or 100×2.1 mm i.d. poly(etheretherketone) (PEEK) column blanks. Chromatograms showing separations of the test mixture including toluene, uracil and cytosine on TRIS-WAX and TRIS-Amide using isocratic elution with 80:20 % (v/v) acetonitrile:ammonium acetate, \(pH \sim 6.8\) are presented in Figures 31 and 32.
It can be seen that both columns showed good separation of uracil and cytosine, with uracil eluting ahead of cytosine due to its higher hydrophilicity. TRIS-WAX and TRIS-Amide show comparatively high column efficiency (N ~34000 plates/m for uracil and 36000-45000 plates/m for cytosine), almost as twice as bare silica column used as starting material (18900 plates /m for uracil and 26500 plates/m for cytosine) (see Table 3, Paper I and Table 7, Paper II). This can be rationalized by faster kinetics of the interaction of solutes with the polymer grafted stationary phases than with the rigid surface of un-functionalized silica. In terms of retention, $k'$ of both uracil and cytosine on the TRIS-WAX phase are larger (almost twice compared to those obtained on bare silica (see Table 3, Paper I). On the TRIS-Amide phase, there was a substantial increase in $k'$ for uracil and cytosine (2.3 to 3.0 times higher compared to those obtained on the TRIS-WAX phase; see Table 7, Paper II). Under these isocratic elution conditions, uracil and cytosine are neutral, therefore their interactions with the polar phases are expected to be solely based on hydrophilic partitioning. The longer retention of the neutral bases on the TRIS-WAX phase than on bare silica indicates an increased hydrophilicity attributed to the tris(hydroxymethyl) and secondary amine groups of the TRIS-WAX stationary phase. This hydrophilicity is even higher on the amide-bonded silica TRIS-Amide phase. This assumption is supported by a correlation between the total amount of grafted polymer on the TRIS-Amide phase being 13.8 % C and 2.2 % N (see Table 3, Paper II), which is a substantially higher amount compared
to the 5.5 % C and 0.55 % N on the TRIS-WAX phase (Paper I, Table 2) based on grafting-specific elements measured by elemental analyses.

**Figure 33.** Chromatogram showing the separation of toluene, uracil and cytosine on 100×2.1 mm i.d. columns packed with Si-BisA, Si-BisA-pMPC and ZIC-chILIC particles. Eluent: 80:20 % (v/v) acetonitrile: 5 mM ammonium acetate, pH ~6.8. Eluent flow rate 0.2 mL/min, injection volume 1.2 µL, UV detection at 254 nm. [Paper IV]

For the Si-BisA and the stratified zwitterionic Si-BisA-pMPC phases in Paper IV, chromatograms showing their separation of the nucleic bases are in Figure 33 shown together with a commercially available polyphosphorylcholine column (ZIC-chILIC), run under isocratic elution condition identical to those used TRIS-WAX and TRIS-Amide columns presented in Figure 31+32.

It can be seen that both uracil and cytosine had substantially higher retention and were better separated on both zwitterionic phases Si-BisA-pMPC and ZIC-chILIC, compared to the Si-BisA column, where the surface is covered only by the strongly crosslinked poly(bisacrylamide) layer designed to shield the silica surface. In terms of efficiency (N), the Si-BisA-pMPC column showed values of 20100 N/m for uracil and 22500 N/m for cytosine, which are comparative to those on bare silica, but lower by about half an order of magnitude compared to those obtained on the commercial ZIC-chILIC phase (Paper IV, Tables 5 and 6). This lower separation efficiency on the Si-BisA-pMPC column could be due to a slower mass transport of the basic solutes with the diblock grafted layers on the Si-BisA-pMPC phase compared to the tentacle layer on the ZIC-chILIC phase [59]. This slow mass transport could indirectly be linked to a higher polymer graft amount (and hence interactive layer thickness) on the Si-BisA-pMPC phase with a loading of 11.2 % C, compared
to 8.6 % C on the ZIC-chILIC material (elemental analysis results; Paper IV, Table 2).

It was also noticed that the higher efficiency of the ZIC-chILIC column could be due to the use of a silica substrate with smaller particle and pore sizes (3 µm and 100 Å) compared to the silica substrate of 5 µm particle size and 200 Å pore size used to prepare the Si-BisA-pMPC phase. The retention factors of uracil and cytosine on the Si-BisA-pMPC phase were slightly lower compared to ZIC-chILIC phase (see Paper IV, Tables 5 and 6). Recall that the Si-BisA-pMPC phase was synthesized using a substrate that is different from the ZIC-chILIC column, i.e. the Si-BisA substrate, and hence the hydrophobic methylene (−CH₂−) moiety of the grafted BIS layer could make the Si-BisA particles become more hydrophobic than the naked silica substrate of the ZIC-chILIC and eventually make the stratified Si-BisA-pMPC phase less hydrophilic compared to ZIC-chILIC. It was noticed that the selectivity of the uracil and cytosine pair (α_{Cyt/Ura} = 2.4) obtained on the Si-BisA-pMPC column was slightly higher than that on ZIC-chILIC column (α_{Cyt/Ura} = 2.2) in 80:20 % (v/v) acetonitrile:5 mM ammonium acetate, pH ~6.8. The less resolved separation between uracil and cytosine on Si-BisA compared to the stratified Si-BisA-pMPC and ZIC-chILIC phases (Figure 33) and the lowest retention factors of uracil and cytosine on this Si-BisA phase among the tested columns (Paper IV, Tables 5 and 6) further hint at the apparent hydrophobicity of the Si-BisA column as a cause for its low retention.

7.2. Separation of Nucleotides

In Paper I, since the TRIS-WAX material contains secondary amine functionality, we expected an anion exchange interaction of this positively charged stationary phase with negative charged solutes under HILIC conditions. A mixture of highly hydrophilic and negatively charged compounds such as nucleotides was chosen as test probes for the TRIS-WAX phase. A mixture of three negatively charged nucleotides including adenosine-5'-mono-, di-, and triphosphate (AMP, ADP, ATP), and its nucleoside base adenosine was injected on the TRIS-WAX column using 70:30 % (v/v) acetonitrile/40 mM ammonium acetate, pH 6.8 as eluent. The four nucleotides were well separated, as shown in Paper I, Figure 7. Adenosine was least retained and the retention increased with the length of the oligophosphate groups in the nucleotides, verifying the mixed HILIC/anion exchange character of the TRIS-WAX column.
7.3. Separation of Organic Acids

A mixture of benzoic acid, acetylsalicylic acid, and four mono-, di-, and trihydroxylated benzoic acid analogs was used for the chromatographic tests of the synthesized materials under HILIC conditions (Papers I-II and IV). In Papers I and II, chromatograms showing the separation of the acid mixture including benzoic, salicylic, acetylsalicylic, 4-hydroxybenzoic, and 3,4-dihydroxybenzoic acids on the TRIS-WAX and TRIS-Amide columns are shown in Figures 34 and 35, respectively.

![Chromatograms showing the separation of the acid mixture](image)

Figure 34+35. Separation of mixtures of five aromatic acidic compounds on columns packed with TRIS-WAX (34, left) and TRIS-Amide (35, right). Column dimensions, eluents, and other conditions as in Figure 31+32. [Papers I and II]

It can be seen that the five benzoic acids are well separated on both TRIS-WAX and TRIS-Amide columns. In terms of retention capacity, these benzoic acids were considerably more retained on TRIS-WAX than on TRIS-Amide (Paper I, Table 4 and Paper II, Table 8). In an eluent made up with unbuffered aqueous ammonium acetate (pH ≈ 6.8), the carboxylic groups of all five acids should be practically fully deprotonated to the anionic carboxylate form (aqueous pKₐ values are listed in Paper I, Table 4), which interact electrostatically with the likewise largely protonated secondary amine functionalities on TRIS-WAX in a mixed HILIC/WAX mode.

On the contrary, TRIS-Amide, which does not contain ionizable groups other than unreacted residual silanols, the retention of these negatively charged benzoic acids was expected to be hydrophilic partitioning and electrostatic repulsion, the latter interaction is most likely with the dissociated residual silanols and at the higher of the tested pH values. The retention of the acids on both the TRIS-WAX
and TRIS-Amide columns increased in the order salicylic acid< acetylsalicylic acid< benzoic acid< 4-hydroxybenzoic acid< 3,4-dihydroxybenzoic acid. This elution pattern is intuitively consistent with an increasing hydrophilicity of the acids, i.e., hydrophilic partitioning accounting for most of the retention mechanism for the acidic solutes. On the other hand, intramolecular hydrogen bonding in ortho-substituted aromatic acids, which reduces the overall polarity of the compound, made salicylic acid and acetylsalicylic acid elute ahead of benzoic acid [Papers I-II]. In Paper II, the addition of 2,6-dihydroxybenzoic acid and 3-hydroxybenzoic acid to the chromatographic test on the TRIS-Amide phase further illustrated that strong intramolecular hydrogen bonding has a profound effect on the apparent solute hydrophilicity in HILIC mode. The electrostatic repulsion effect which increases with decreasing pKₐ values of the acids made 3-hydroxybenzoic acid elute ahead of 4-hydroxy benzoic acid. The elution pattern on TRIS-Amide was consequently 2,6-dihydroxybenzoic acid< salicylic acid< acetylsalicylic acid< benzoic acid< 3-hydroxybenzoic acid< 4-hydroxybenzoic acid< 3,4-dihydroxybenzoic acid (see Paper II, Figure 7).

In Paper IV, the same test mixture of seven benzoic acids used on the TRIS-Amide column was tested on the stratified zwitterionic Si-BisA-pMPC phase, alongside the Si-BisA and commercial ZIC-cHILIC columns, using 80:20 % (v/v) acetonitrile:20 mM ammonium acetate, pH ~6.8 as eluent (Figure 36). It can be seen that the seven benzoic acids were best resolved on Si-BisA-pMPC, with the elution order following the pattern observed on the TRIS-WAX and TRIS-Amide phases – again pointing at hydrophilic partitioning as the main retention mechanism on Si-BisA-pMPC under these elution conditions (Paper IV). The Si-BisA and ZIC-cHILIC phases also showed similar elution patterns for the acidic mixtures as the Si-BisA-pMPC column. The Si-BisA column could not provide a baseline separation for the seven acids, which could be due to an insufficient hydrophilicity of this stationary phase, as mentioned earlier. On the ZIC-cHILIC column, acetylsalicylic acid and benzoic acid coeluted.

It was noticed that the elution order of the acids was different on the bare silica with 2,6-dihydroxybenzoic acid< salicylic acid< benzoic acid< 3-hydroxybenzoic acid< acetylsalicylic acid< 4-hydroxybenzoic acid< 3,4-dihydroxybenzoic acid (Figure 36). Further effects of different mobile phase conditions, e.g., the mobile phase pH and salt concentration, will be discussed in the parts below to unravel the selectivities offered by these three synthesized materials.
Figure 36. Separation of a mixture of seven aromatic acids on columns packed with Kromasil silica, Si-BisA, Si-BisA-pMPC and ZIC-chILIC particles. Eluent, 80:20 % (v/v) acetonitrile:20 mM ammonium acetate, $pH \approx 6.8$. Column dimensions 100×2.1 mm i.d., eluent flow rate 0.2 mL/min, injection volume 1.2 µL, UV detection wavelength 254 nm. The peak identities are as follows; 1, 2,6-dihydroxybenzoic acid; 2, salicylic acid; 3, acetylsalicylic acid; 4, benzoic acid; 5, 3-hydroxybenzoic acid; 6, 4-hydroxybenzoic acid; 7, 3,4-dihydroxybenzoic acid. [Paper IV]

7.4. Column selectivity

In order to get an overview on the retention mechanism of the TRIS-WAX, TRIS-Amide and the Si-BisA-pMPC phases, different mobile phase parameters including $pH$ and salt concentration have been varied.

7.4.1. $pH$ effect

The effect of the mobile phase $pH$ on the retention of the tested compounds used in Papers I and II, including nucleic bases, positively charged amine (ephedrine) and negatively charged benzoic acids, on TRIS-WAX and TRIS-Amide columns, is shown in a polar diagram (Figure 37). It reveals similar $pH$ effects on both phases for the nucleic bases and the strong base ephedrine, i.e., the retention factors for
uracil and cytosine remained essentially constant when the eluent was changed from unbuffered eluent pH~ 6.8 to buffered eluent pH 4.0 while the retention of ephedrine was reduced, most substantially on the TRIS-WAX column under 5 mM salt concentration on both columns.

When the benzoic acids were subjected to the same pH change in the eluent, the retention times of most of the aromatic acids decreased on both stationary phases (Paper I, Table 4 and Paper II, Table 8) due to decreased polarities of the acids at the lower pH 4.0. Opposing this trend were the hydroxylated benzoic acids with propensity for intramolecular hydrogen bonding (2,6-dihydroxybenzoic acid and salicylic acid), the retention of which increased slightly when the mobile phase pH decreased on both columns. This is attributed to a lower extent of intramolecular hydrogen bonding, causing these acids to express higher hydrophilicity in their protonated state at lower pH, as discussed above. The elution order of the seven benzoic acids on the TRIS-Amide column at pH 4.0 was 2,6-dihydroxybenzoic acid< salicylic acid< 4-hydroxybenzoic acid< benzoic acid< acetylsalicylic acid< 3-hydroxybenzoic acid< 3,4-dihydroxybenzoic acid (Paper II, Table 8). This elution order for the acids at pH 4.0 was not the same as that obtained on the TRIS-WAX column with 4-hydroxybenzoic acid< benzoic acid< salicylic acid< acetylsalicylic acid< 3,4-dihydroxybenzoic acid, where the retention times increased with decreasing aqueous pK_a values of the acids under influence of the anion exchange contribution to the retention mechanism (see Paper I, Figure 6).
**Figure 37.** Polar diagram of retention factors ($k'$) of TRIS-Amide and TRIS-WAX phases. Legend: U, Uracil; C, Cytosine; E, Ephedrine; S, Salicylic acid; B, Benzoic acid; A, Acetylsalicylic acid; H, 4-Hydroxy-benzoic acid; D, 3,4-Dihydroxybenzoic acid. Solid lines correspond to pH 4.0 and dashed lines to pH 6.8. [Paper II]

In **Paper IV**, the mobile phase pH effect on the retention pattern was determined by an extended solute set encompassing fourteen different compounds, including neutral nucleic bases and amino acids, positively charged amines, and negatively charged benzoic acids. The column tested was Si-BisA-pMPC, using the commercial ZIC-chILIC column as benchmark; results shown in Figure 38.
Figure 38. Polar diagram of probe retention factors ($k'$) on Si-BisA-pMPC and ZIC-chILIC phases.
Legend: U, uracil; C, cytosine; E, ephedrine; Ep, epinephrine; NE, norepinephrine; 2,6-HB, 2,6-dihydroxybenzoic acid; S, salicylic acid; B, benzoic acid; A, acetylsalicylic acid; 3-HB, 3-hydroxybenzoic acid; 4-HB, 4-hydroxybenzoic acid; D, 3,4-dihydroxybenzoic acid; Tryp, Tryptophan; Tyr, Tyrosine. Solid lines correspond to $pH$ 4.0 and dashed lines to $pH$ 6.8. [Paper IV]

When changing from unbuffered eluent $pH \approx 6.8$ to buffered eluent $pH$ 4.0, the retention times of uracil and cytosine hardly changed on both zwitterionic columns. Negligible changes in retention were also seen for the two amino acids tryptophan and tyrosine on both stationary phases as response to the same $pH$ change, even though a slight retention decrease was evident at the higher salt concentration 20 mM on the ZIC-chILIC column. Under the used mobile phase $pH$s, the nucleic bases and the amino acids are all neutral/zwitterionic (cf. aqueous $pK_a$ values in Papers I-III), and the two zwitterionic stationary phases have nominally $pH$-independent charges, thus the retention mechanism of the neutral compounds should essentially be based on hydrophilic partitioning alone.

For the strong bases ephedrine, epinephrine, and norepinephrine, the retention decreased with decreasing mobile phase $pH$ and this decrease was stronger at the higher electrolyte concentration. For these strongly basic compounds, whose $pK_a$ values are above 9.8 (cf. Paper III), they existed as protonated amines at both $pH$ 6.8 and 4.0, which makes them interact with the dissociated residual silanols on the Si-BisA-pMPC and ZIC-chILIC via electrostatic interaction. For the mixture of the seven benzoic acids and analogs, the retention time decreased substantially on both zwitterionic stationary phases when the mobile phase $pH$ was decreased,
which is due to the decreased polarities of the protonated acids, as mentioned in Papers I and II.

Opposed to this trend on both columns were 2,6-dihydroxy benzoic acid and salicylic acid, which have intramolecular hydrogen bonding effect, the retention of which increased when the mobile phase pH was decreased. This can again be due to the lesser extent of intramolecular hydrogen bonding effects in the 2,6-dihydroxybenzoic acid and salicylic acid as mentioned above. The elution order of the acidic mixture at pH 4.0 on both Si-BisA-pMPC and ZIC-cHILIC stationary phases were similar with 2,6-dihydroxybenzoic acid< benzoic acid< acetylsalicylic acid and 4-hydroxybenzoi acid and salicylic acid< 3-hydroxybenzoic acid< 3,4-dihydroxybenzoic acid (see Paper IV, Figure 8). This elution pattern is different from that seen on the neutral TRIS-Amide phase (Paper II). Both on the neutral TRIS-Amide and on the zwitterionic phases, the elution order for the acids at pH 4.0 showed the hallmarks of a mixed HILIC/electrostatic repulsion retention mechanism. The difference in this elution pattern between the supposedly neutral TRIS-Amide and the zwitterionic Si-BisA-pMPC as well as ZIC-cHILIC phases can be due to the difference in relative contribution of each interaction mechanism to a mixed HILIC/electrostatic repulsion retention mechanism on each of the tested columns. This difference might be related both to the differences in surface charge density of the TRIS-Amide and the Si-BisA-pMPC, characterized by ζ-potential measurements as mentioned above (Tables 2 and 3), and to specific solute-stationary phase interactions.

7.4.2. Electrolyte concentration effects

Both the concentration and nature of the (buffer) electrolyte is known to have a pronounced effect on retention in HILIC\textsuperscript{64}. The choice of salts to use as buffer compounds is, however, rather limited – the primary criteria that need to be fulfilled by a buffer substance in HILIC are a) solubility in water/acetonitrile mixtures at water admixtures as low as 5%; and b) buffering activity over a wide pH range. Since HILIC has gained tremendous popularity as separation method coupled to electrospray mass spectrometry (ESI-MS), compatibility with this ionization and detection mode is also desirable when methods are developed and when the selectivity of new columns are assessed. The choice of eluents is consequently very limited, and is basically restricted to ammonium salts of small carboxylic acids, in particular ammonium acetate and formate. Buffers that perform well in the neutral pH range and that are compatible with ESI-MS are not readily at hand.
In order to carry out evaluations under eluent conditions that are close to realistic use situations, we decided to settle for isocratic elution at 80:20 % (v/v) acetonitrile/water with ammonium acetate as buffer at final concentrations 5 and 20 mM. Two pH levels were chosen, pH 4.0 (where this salt acts as an actual buffer after pH adjustment with acetic acid), and pH ≈ 6.8, which is the value measured when ammonium acetate is dissolved in water. Admittedly, this is far away from the optimal buffer range for ammonium acetate in aqueous solution, although at high acetonitrile concentrations the acid dissociation constants of both acetic acid (the corresponding acid of the acetate ion) and the ammonium ion are moved closer to neutral because of the limited ability of low permittivity solvents to stabilize ions by solvation. The choice of buffer substance, its concentration and pH is therefore a reasonable compromise between establishing reproducible tests and realistic conditions for users of the primary detection technique used in HILIC.

A typical HILIC behavior involves a retention increase with increasing electrolyte concentration in the mobile phase\textsuperscript{157,158}. A polar diagram showing a comparison of the ionic strength effects on TRIS-Amide and TRIS-WAX is presented in Figure 39. On the TRIS-Amide column, an increase in the electrolyte concentration in the eluent from 5 to 20 mM results in increased retention for all the aromatic acids, and to a lesser extent also for the neutral solutes uracil and cytosine. This is consistent with the hydrophilic partitioning retention mechanism discussed above.

For the basic solute ephedrine, the increase in eluent electrolyte concentration caused a reduced retention which was most prominent at pH 6.8 and is explained by reduced cation exchange interaction between silanolate groups and the positively charged ephedrine at increased salt concentration. On TRIS-WAX, increased retention was also seen for the neutral bases uracil and cytosine when the electrolyte concentration was increased from 5 to 20 mM, which is again due to the increased hydrophilicity of the stationary phase. Opposite effects of electrolyte concentration on the retention of the aromatic acids and ephedrine were seen on the TRIS-WAX phase compared to those on TRIS-Amide, as mentioned above; an increased electrolyte concentration led to decreased retention for the aromatic acids, which is explained by shielding of the electrostatic attraction between the negatively charged acids and the positively charged amine groups on the TRIS-WAX phase by acetate ions. For ephedrine this increased retention is attributed to reduced electrostatic repulsion between ephedrine and the TRIS-WAX phase, bearing the same charge sign.
Figure 39. Polar diagram of retention factors (k’) of TRIS-Amide and TRIS-WAX phases. Legend: U, Uracil; C, Cytosine; E, Ephedrine; S, Salicylic acid; B, Benzoic acid; A, Acetylsalicylic acid; H, 4-Hydroxy-benzoic acid; D, 3,4-Dihydroxybenzoic acid. Solid lines correspond to pH 5 mM and dashed lines to 20mM. [Paper II]

In Paper IV, the ionic strength effects on the test solute retention was assessed on Si-BisA-pMPC and compared to the commercial ZIC-cHILIC phase; see Figure 40. When the mobile phase salt concentration was increased from 5 to 20 mM, the retention of the neutral nucleic bases and amino acids, and the negatively charged benzoic acids increased on both columns, which is consistent with the hydrophilic partitioning retention mechanism, as discussed above. Opposing this trend on both columns was the strong bases ephedrine, epinephrine, and norepinephrine, which retentions decreased at elevated the electrolyte concentration 20 mM. This can be due to the increased shielding effect at higher salt concentration, causing a decreased electrostatic interaction between positively charged amines and negatively charged residual silanolates on both stationary phases. In conclusion the synthesized zwitterionic Si-BisA-pMPC phase behaved similarly to the ZIC-cHILIC column under the investigated salt concentrations.
Figure 40. Polar diagram of retention factors ($k'$) of Si-BisA-pMPC and ZIC-chILIC phases. Legend: U, Uracil; C, Cytosine; E, Ephedrine; Ep, Epinephrine; NE, Norepinephrine; 2,6-HB, 2,6-dihydroxybenzoic acid; S, Salicylic acid; B, Benzoic acid; A, Acetylsalicylic acid; 3-HB, 3-Hydroxybenzoic acid; 4-HB, 4-Hydroxy- benzoic acid; D, 3,4-Dihydroxybenzoic acid; Tryp, Tryptophan; Tyr, Tyrosine. Solid lines correspond to 5 mM and dashed lines to 20 mM. [Paper IV]

7.4.3. Selectivity comparison with commercial HILIC columns [Paper III]

To extend the selectivity characterization and comparison of the TRIS-WAX and TRIS-Amide stationary phases, Paper III addressed a more detailed investigation of pH and salt effects on the selectivity of these two TRIS-based phases relative to two commercially available polymer grafted/coated silica-based HILIC phases, i.e. ZIC-HILIC (polysulfoalkylbetaine) and Luna Diol (polyol). Kromasil silica was also included into the test, since it was used as substrate for the two TRIS materials.

Nowadays the most common systems of stationary phase property characterization in HILIC are based on application of tests based on a certain number of solutes resulting in a specification of the retention behavior of those tested solutes. One should keep in mind that the properties found in this way may differ when a different set of solutes is used to test the same stationary phase. We used a set of ten different solutes of acidic, basic and neutral characters as model compounds as shown in Paper III, Figure 1. An extended series of different electrolyte concentrations with ammonium acetate/ acetic acid bufferpH 4.0 and ammonium acetate pH ≈ 6.8, both at final ammonium ion concentrations of 5, 10, 20 and 50 mM in the mobile phase, was therefore used at unaltered acetonitrile:water ratio to evaluate the for HILIC mode retention pattern. With this, we hoped to find a more stringent
test to describe the selectivity dimensions of our two synthesized TRIS phases, in comparison to two commercial HILIC stationary phases, as function of electrolyte concentrations. The relative retentivity of ten test solutes on the five stationary phases under these conditions is shown in Figure 41.

![Figure 41](image)

**Figure 41.** Retention factors of ten tested solutes on the five polar columns. Flow rate: 0.2 ml/min. Mobile phase: acetonitrile/water 80/20 % (v/v) containing 5 mM ammonium acetate. Legend: B, benzoic acid; HB, 3-hydroxybenzoic acid; DHB, 3,4-dihydroxybenzoic acid; THB, 3,4,5-trihydroxybenzoic acid; Tryp, tryptophan; Tyr, tyrosine; DOPA, (2S)-2-amino-3-(3,4-dihydroxyphenyl)propanoic acid; NE, norepinephrine; EH, ephedrine; EN, epinephrine. [Paper III].

In brief, it can be seen that for the four leftmost tested solutes, the benzoic acids, TRIS-WAX had the highest retention, rationalized by the strong contribution of the anion exchange interaction in a mixed HILIC/WAX retention mechanism. For the three neutral amino acids, the TRIS-Amide showed the highest retention. Under the mobile phase condition of the test, the neutral amino acids interacted with the polar phases via hydrophilic partitioning, as discussed above. The high retention expressed by the TRIS-Amide phase for these neutral polar compounds hints at the TRIS-Amide phase as providing the highest hydrophilicity-mediated partitioning. This high capability of offering HILIC partitioning type interaction could be due to high amount of polar polymer grafting on TRIS-Amide (13.8% C by elemental analysis, cf. Paper II). Among the four polymer-functionalized silica phases, Luna- Diol provided the lowest retention for the amino acids, which indicates a low total amount of hydrogel-forming organic phase on Luna Diol. Bare silica also showed the low retention factors for the amino acids, comparable to the Luna Diol, irrespective of the difference in chemical functionalities on these two phases. For the strongly basic test probes(ephedrine, epinephrine, and norepi-
nephrine), TRIS-WAX was the least retentive phase, attributed to electrostatic the repulsion between these positively charged amines bearing the same charge sign as the protonated secondary amine of the alkyl-linked TRIS moieties.

Among the remaining columns, four expressed negatively charged surfaces (as characterized by \( \zeta \)-potential measurements as above); ZIC-HILIC showed the highest retention for the basic compounds (except for ephedrine, for which TRIS Amide still offered the strongest retention), followed by TRIS-Amide, silica, and Luna Diol. These phases expressing negative surface charges under the evaluated HILIC conditions will interact electrostatically with the positively charged bases. However, the higher retention of the bases on ZIC-HILIC and TRIS-Amide than on silica and Luna Diol phases can only be due to a higher hydrophilicity mediated by partitioning than that offered by bare silica and Luna Diol. The retention factors of the basic analytes on the Luna Diol phase were low and comparable to the TRIS-WAX phase, which indicates limited electrostatic interaction between the strong bases and the dissociated residual silanol groups on Luna Diol. The low silanol activity on Luna Diol is consistent with results reported by Lindner \textit{et al.}\textsuperscript{64}

Detailed effects of mobile phase pH and electrolyte concentration on the retention pattern of ten selected test solutes on the five polar phases are individually presented in \textbf{Paper III}, Figures 4-8. In conclusion, by applying the set of model compounds comprising acidic, basic and neutral/zwitterionic analytes on our two newly synthesized TRIS-based phases and two commercial polymer grafted silica HILIC phases together with bare silica under different pHs and salt concentrations in the HILIC eluents, we could clearly see the distinct selectivity of our two synthesized stationary phases in relation to the other three phases as follows:

- Increasing the mobile phase pH generally increased the retention of all tested solutes on all five tested silica-based materials, regardless of substantial differences in chemical surface functionality. This is most likely due to dissociation of the (residual) silanol groups at the higher pH \( \approx 6.8 \), which made the phases more polar. In terms of elution pattern, a decrease in mobile phase pH from 6.8 to 4.0 altered the elution order of the benzoic acids on the TRIS-WAX column under a mixed anionic exchange interaction/hydrophilic partitioning mechanism, while hydrophilic partitioning remained dominant for the retention of the acids on other ZIC-HILIC, TRIS-Amide, and Luna Diol phases. The elution pattern of the benzoic acids seemed to follow an electrostatic repulsion mechanism on bare silica in a mixed electrostatic repulsion/hydrophilic
partitioning, *i.e.*, benzoic acids with lower pKₐ values eluted earlier at both tested pHs in the mobile phase.

- Upon increasing electrolyte concentration in the mobile phase from 5 to 50 mM:

  (a) Silica demonstrated hydrophilic partitioning and electrostatic repulsion with the acidic compounds. For the zwitterionic amino acids, hydrophilic partitioning was the dominant mechanism. Bare silica showed a very different selectivity for the basic test solutes compared to the polymer-functionalized silica phases: At 5-20 mM ammonium ion concentration the elution appeared to follow weak cation exchange interaction, whereas at 50 mM at both tested pHs, hydrophilic partitioning became dominating and hence the elution pattern of the strongly basic test solutes appeared to follow a hydrophilic partitioning as on the four polymer-functionalized silica phases. The retention factors of the three strong bases were reduced at the lower pH of 4.0 and at 5-20 mM ammonium ion concentration at pH ≈ 6.8, due to weak cation exchange interaction. However, at 50 mM ammonium acetate and pH ≈ 6.8, the cation exchange interaction between the silanols and the protonated bases was largely suppressed and hydrophilic partitioning came into play, as revealed by higher retention of the bases at this condition than those at 20 mM salt concentration, pH ≈ 6.8.

  (b) TRIS-WAX demonstrated both weak anion exchange and hydrophilic interactions for the acidic and neutral amino acids, and it also showed electrostatic repulsion interaction with the strongly basic test solutes. At 50 mM ammonium acetate, pH ≈ 6.8, hydrophilic partitioning became dominant and suppressed the anion exchange interaction for the acidic compounds, causing an increase in the retention of all acids compared to 20 mM ammonium acetate. The retention factor of 3,4,5-trihydroxybenzoic acid even exceeded the value obtained at the lowest ammonium acetate concentration of 5 mM.

  (c) TRIS Amide expressed a dominant hydrophilic partitioning for acids and amino acids. A weak cation exchange pattern was observed for ephedrine over the entire ammonium ion concentration span, from 5 to 50 mM. Weak cation exchange was also seen for the other two bases, norepinephrine and epinephrine in the ammonium ion concentration interval 5-20 mM; while at 50 mM ammonium acetate in the eluent, hydrophilic partitioning became dominant causing a re-increase in the retention for these two bases.
(d) The retention pattern of ZIC-HILIC was dominated by hydrophilic partitioning for the acids and the amino acids. Some cation exchange interaction was seen for all three tested bases over the entire ammonium ion concentration range at pH 4.0. At pH ≈ 6.8, cation exchange interaction was seen for the three bases at 5-20 mM, whereas at 50 mM hydrophilic interaction became dominant over cation exchange, as observed on the TRIS-Amide phase.

(e) Luna Diol seemed to be dominated by hydrophilic partitioning for all tested solutes, except for ephedrine. For this solute, the retention hardly changed at pH 4.0 and in the 5-20 mM ammonium ion concentration range at pH 6.8. At 50 mM and pH 6.8, hydrophilic partitioning became dominant and made the retention of ephedrine increase substantially. This phase has the least silanol activity effect on the retention of basic compounds among the tested silica-based stationary phases. However, its overall retention is also low.

8. Long-term Column Precision

It was interesting to assess the long-term stability of the two TRIS-based phases in terms of retention time reproducibility under HILIC elution conditions. On the TRIS-WAX column, the retention times of uracil and cytosine were recorded at room temperature using as mobile phase 80/20 % ACN/5mM ammonium acetate, pH ≈ 6.8 at a flow rate of 0.15 mL/min. Injection volume 1.2 µL and UV detection at 254nm. After two years, the retention factor of uracil had reduced from 0.55 to 0.45 and that of cytosine from 1.36 to 0.91. During this time period, the TRIS-WAX column had alternately been in use and kept stored in the fridge (~8°C) in HILIC eluents when not in use. This reduction in retention times of uracil and cytosine could indicate a phase loss during this period of time, which can be due to an incomplete washing to remove free oligo/polymers that were not covalently bound but adsorbed to the silica surface of the TRIS-WAX particles after the synthesis step. This may cause the drift in the retention times of uracil and cytosine by time due to the continuous phase ‘washing’ when the column was running with HILIC eluents. On the other hand, a polymeric secondary amine silica phase like TRIS-WAX could be subject to hydrolysis after a long use time. This hydrolysis seems to be related to a catalytic self-predatory action of the primary amino group on the ligand bonded to silica, which is often seen on traditional monolayer-functioned primary aminopropyl silica and causes fast release of ligands together with accompanying peak shape deterioration under HILIC conditions. This phase strip
(to a lesser extent) phenomenon could also have happened on the TRIS-WAX stationary phase due to catalytic reaction of the secondary amines of the terminal TRIS groups from the polymeric side chains with the ligands bonded to silica surface.

On the TRIS-Amide column, the retention of uracil and cytosine mixture was recorded periodically using conditions and mobile phase as above, albeit at a flow rate of 0.2 mL/min. Approximately 600 analyses were performed on the column packed with TRIS-Amide particles, and standard deviation of the retention factor values ($k'$) never exceeded 0.02 for uracil and 0.03 for cytosine. This indicates a good column stability of the TRIS-Amide phase.

9. Concluding Remarks and Future Aspects

High grafting yield could be obtained by different “grafting from” polymerization techniques that were used in this thesis, revealed by the total carbon content on the grafted silica particles analyzed by elemental analysis from 5.5 % to 13.8 % at polymerization times as short as 6-18 hours. Among the “grafting from” polymerization techniques, grafted silica synthesized by “controlled/living” radical polymerizations like ATRP and photoiniferter mediated polymerization showed less reduction in surface area and pore volume due to a slower polymerization speed compared to the degree of pore filling obtained by conventional free radical polymerization [Papers I-II and IV]. Conventional free radical polymerization based on tert-butyl peroxide group attached to silica surface proved to be robust and suitable for polymerizing highly hydrophilic and amphiphilic monomer like TRIS-Acrylamide in a partly aqueous solvent [Paper II]. “Controlled /living” radical polymerizations can be used interchangeably depending on the monomer functionality. Photoiniferter-mediated polymerization from $N,N$-diethylldithiocarbamate groups immobilized silica proved to be robust for block graft copolymerization of different hydrophilic monomers onto the silica surfaces [Paper IV] and thus makes stationary phase architecture design with layered polymeric functionalities possible.

In terms of chromatographic selectivity of the newly introduced stationary phases for HILIC, we succeeded in synthesizing a pair of TRIS-based columns having distinct and rather orthogonal/complementary selectivities to each other [Papers I-III]. The TRIS-WAX column showed a distinct anion exchange character in a mixed hydrophilic interaction/WAX mode. The TRIS-Amide column showed a
similar adsorption characteristic with dominant hydrophilic partitioning, and lacked the anion exchange character of the TRIS-WAX column. Column stability under aqueous HILIC eluents of the amino based TRIS-WAX stationary phase can be improved by applying the same chemical modification method on polymeric substrates, both particulate and monolith, or on organic-silica hybrid substrate like bridged ethylene hybrid (BEH) supports were surface silanol groups are substantially reduced and the pH stability range is extended to 1<pH<8.70.

The stratified bis(acrylamide)/polyphosphorylcholine grafted silica introduced in this work proved to be useful for HILIC application and possessed a selectivity that was quite different from to the commercially available non-stratified polyphosphorylcholine phase ZIC-cHILIC [Paper IV]. On the other hand, this stratified material, with a highly crosslinked poly(bisacrylamide) “undercarpet”, is expected to be more stable against hydrolysis, which often plagues bonded silica stationary phases in aqueous HILIC eluents. The enhanced column stability of the stratified material remains to be tested by synthesizing the same polyphosphorylcholine grafted silica phase without the cross-linked BIS layer underneath by the method described in Paper IV and comparing long-term column stability with the current synthesized stratified material.
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11. References


