Zwitterionic Sulfobetaine Polymers as Stationary Phases for Liquid Chromatography

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Abstract
Liquid chromatography is an important separation technique for a vast number of analytes. This thesis mainly focuses on the development of stationary phases for liquid chromatography based on zwitterionic sulfobetaine polymers.

In the thesis, several ways to prepare zwitterionic polymers in an aqueous environment using reversible addition fragmentation chain transfer (RAFT) polymerization are described. Both telomers, i.e. short soluble polymer chains containing a functional terminal group, as well as graft polymers have been synthesized. The RAFT polymerization technique provides an increased degree of control of the final polymers, which may aid in the preparation of more specifically tailored separation materials.

Sulfobetaine polymers carry both a positive and a negative charge within a single entity, which results in interesting solution properties as well as highly biocompatible features. These unique features make them especially suited for separation of highly polar and/or charged compounds. An example of the successful separation of short peptides using a stationary phase synthesized with the RAFT technique is given.

The unusual properties of sulfobetaine-type polymers are believed to be associated with the structure of water close to the polymer. An NMR study of water structure in bare silica as well as silica grafted with zwitterionic sulfobetaine polymers was conducted. The grafted silica samples contain a larger fraction of unfrozen water at temperatures well below 0 °C compared to the bare silica, which is believed to be associated with the retention characteristics of these materials.

Keywords: sulfobetaine, polymer, RAFT polymerization, zwitterionic, separation, liquid chromatography, stationary phase

“He who never made a mistake never made a discovery”

Samuel Smiles
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Abbreviations

ACN  Acetonitrile
ASEC  Aqueous size exclusion chromatography
ATRP  Atom transfer radical polymerization
CTA  Chain transfer agent
DLS  Dynamic light scattering
DVB  Divinylbenzene
EIC  Electrostatic ion chromatography
ESI-MS  Electrospray ionization mass spectrometry
GFC/GPC  Gel filtration/permeation chromatography
GPEC  Gradient polymer elution chromatography
HILIC  Hydrophilic interaction chromatography
HPLC  High performance liquid chromatography
IEC  Ion exchange chromatography
M  Monomer
MALDI  Matrix assisted laser desorption/ionization
TOF  Time-of-flight
MPC  2-Methacryloyloxyethyl phosphorylcholine
NMP  Nitroxide mediated polymerization
NMR  Nuclear magnetic resonance
ODS  Octadecylsilica
PDI  Polydispersity index
QCM-D  Quartz crystal microbalance with dissipation monitoring
RAFT  Reversible addition fragmentation chain transfer polymerization
RI  Refractive index
RP  Reversed phase
SAM  Self assembled monolayer
SPE  3-[2-(N-methacryloylethyl)-N,N-dimethylammonio] propane sulfonate
STPE  Sodium 2-(2-thiobenzoysulfanyl propionylamino) ethanesulfonate
XPS  X-ray photoelectron spectroscopy
ZIC  Zwitterion chromatography
List of Papers

This thesis is based on the following manuscripts, which are referred to in the text by their Roman numerals:

I. Synthesis of Sulfoalkylbetaine Zwitterionic Polymers by Aqueous UV-initiated RAFT Polymerization
   Erika Wikberg, Jeroen J. Verhage and Knut Irgum
   *Submitted to Journal of Polymer Science Part A: Polymer Chemistry*

II. Grafting of Silica via Aqueous Reversible Addition Fragmentation Chain Transfer Polymerization and its Use as a Liquid Stationary Phase in Hydrophilic Interaction Chromatography
    Erika Wikberg, Jeroen J. Verhage, Camilla Viklund and Knut Irgum
    *Submitted to Journal of Separation Science*

III. Graft Polymerization of 3-[N,N-dimethyl-N-(methacyloyloxyethyl) ammonium] propanesulfonate from Divinylbenzene Particles by Living Free Radical Polymerization Techniques
     Erika Wikberg, Fredrik Limé, Jeroen J. Verhage and Knut Irgum
     *Manuscript*

IV. A ²H NMR Study of Water in Zwitterionic Stationary Phases and its Influence on Retention Characteristics
    Erika Wikberg, Tobias Sparrman, Camilla Viklund, Tobias Jonsson and Knut Irgum
    *Submitted to Analytical Chemistry*

Also by the author but not included in the thesis

V. Effect of Electrolyte Composition and Concentration on the Response from the Dissipative Quartz Crystal Microbalance
   Erika Wikberg, Meenakshi Singh and Knut Irgum
   *Manuscript*
1. Introduction

“What does this sample contain?” This is one of the most frequently asked questions to an analytical chemist, and in most cases, it is also possible to provide the person with an acceptable answer. More specifically, analytical chemistry is a branch in chemistry that is concerned with questions like "what chemicals are present in this sample, what are their characteristics and how much is really in there?"\(^a\) The ability to characterize samples and determine the amounts of different analytes in them is not restricted to chemistry, but is of great importance in most fields within natural sciences. To those not familiar with chemical vocabulary, the term analyte refers to any of the different compounds or molecules in a sample that is going to be measured. The analysis of drug metabolites in human blood, vitamin C levels in orange juice, detection and quantification of poisonous pesticides like DDT in soil, or the recently debated presence of acrylamide in food, are all examples of problems that require knowledge about analytical chemistry. While other branches of chemistry focus on a specific problem, whether it is synthesis of complex organic compounds or the understanding of intricate biochemical processes in a cell, most scientists depend on a variety of analytical techniques to answer their questions. The research in analytical chemistry focuses on the development of efficient and reliable methods to determine the contents of a sample, regardless of its origin.

To someone not familiar with chemistry in general and analytical chemistry in particular, a common request is “analyze this for me”. Well, by simply looking at a sample it is usually possible to determine if it is a solid or a liquid, metal or organic matter, etc. By observing the color and smell of a substance, it is sometime possible to give a hint of what it contains, for example if it contains strongly smelling compounds like amines or sulfur species. In order to provide a more detailed analysis of a sample, it is however necessary to have at least some clue, not only of what the person is looking for, but also why. Depending on what you want to analyze, everything from sample preparation to the final analysis

need to be considered so that the analyte of interest is not lost or destroyed during any of the sample preparation steps, or unintentionally transformed into another chemical compound through a chemical reaction. All of these factors need to be taken into account in order to obtain a true and reliable answer to the original question.

2. Liquid chromatography

In order to quantify a specific analyte in a sample that contains many different compounds, it is often necessary to isolate the response from this analyte from that of other components in the sample. There are basically two ways to solve this problem, either by using a highly specific detection technique that selectively responds only to the analyte of interest, for example by using an (ideal) immunoassay, or by separating the analytes from other sample components by some separation technique, most of which are based on chromatography. The first approach, to selectively detect only one analyte, may be valuable for certain analytes such as proteins or other large molecules, but there is always a certain risk of non-specific recognition, and false results may thus occur. When more than single analyte in a sample is of interest, a specific method that only measures one compound is obviously not very useful, and a separation combined with a more general detection principle is typically required.

Another aspect of chemical analysis is the detection of the analytes after a separation has occurred. In chromatography, detection techniques with a wide scope such as single wavelength UV/VIS absorption, electrical conductivity, viscosity, or refractive index are often used. These are all general detection techniques that provide a response for many different analytes in a sample, but since they respond to a common physical or chemical property of the analytes in a univariate fashion, they yield no information about the identity of the analyte. To obtain a positive identification of an analyte, a specific detection technique must be used. Today, the dominating technique for this purpose is mass spectrometry, which can provide a positive identification of basically all compounds provided they can be transferred to the gas phase and ionized, and also detailed information through ion fragmentation patterns, which may
yield positive structure identification and give insight into reaction mechanisms.

A simple example of a separation technique is liquid/liquid extraction, in which two immiscible liquids, typically water and an organic solvent, are mixed by vigorous shaking. This technique can be used to separate water-soluble compounds from compounds soluble in organic solvent but it is a rather rough single-step technique that is usually not selective enough for chemical analysis. Liquid chromatography is based on a similar mechanism, namely partitioning of an analyte between two different phases; a stationary phase placed inside a column, and a liquid mobile phase which is flushed through the column. A sample is introduced at one end of the column, and transported through the column by the mobile phase that is constantly pumped through the column. The stationary phase and the mobile phase have different properties, and depending on the properties of a specific molecule, it will prefer to be either in the stationary phase or in the mobile phase. A molecule that prefers to be in the mobile phase will travel through the column quite fast, while a molecule that prefers the stationary phase will spend a longer time in the column. Each analyte will thus exit the column at different times, and can be detected and quantified separately. A scheme of a typical liquid chromatography system is shown in Figure 1.

![Figure 1. Basic setup of a liquid chromatography system. All components are connected via narrow tubing.](image-url)
The objective of this thesis was to develop new and more controlled ways to prepare sulfobetaine-type stationary phases for liquid chromatography, and to get a better understanding of their interaction with various analytes. The most common stationary phase used in liquid chromatography is still silica modified with C_{18}-chains, but for separation of highly polar or charged compounds, as in the case of many biomolecules, other types of stationary phases may be better suited to achieve a good separation.

Examples of biomolecules include proteins (including enzymes), carbohydrates, lipids, phospholipids, sugar derivatives, hormones, steroids, and various smaller peptides. Many of these compounds are water-soluble, and particularly in the case of proteins, their structure and conformation can change if they are heated or subjected to organic solvents. Proteins also tend to adsorb onto surfaces, which often causes them to denature. This is a problem in chromatography, where separation requires close encounter of an analyte with the stationary phase surface. The development of stationary phases that are able to resist protein adsorption and show general biocompatible properties is thus of great interest to applications involving biomolecules, especially in view of the growing interest in proteomics, metabolomics, and other related research areas.

The materials investigated here are based on zwitterionic polymers. The unique properties that these materials provide have shown to be very suitable for separation of hydrophilic compounds and biomolecules. One of the original purposes was to study the interaction between proteins and surfaces covered with zwitterionic polymers, in order to better understand the retention mechanism of proteins on zwitterionic stationary phases. Other objectives included the polymerization of zwitterionic monomers in a controlled way, in order to be able to prepare highly tailored materials with specific properties.

3. Sulfobetaine polymers in chromatography

Sulfobetaine polymers are interesting as separation materials in liquid chromatography because of the presence of both positive and negative ionic groups. The German word “zwitter” means hybrid, associated with
hermaphroditism in biology, and a zwitterion is hence a molecule that incorporates both a positive and a negative ionic group. A common group of zwitterions that many are familiar with is amino acids, although some also contain additional ionic groups other than the amine and carboxylic groups in the aminoacetic moiety. Proteins contain a varying number of positive and negative surface charges, and may actually be considered to be polyzwitterions, although the correct term for this multitude of opposite weak charges that can act both as acids and as bases is amphoteric.

Polymers that bear charged groups can be divided into several categories, depending on the location of the ionic groups within the polymer (Figure 2). Polyelectrolytes are perhaps the most well-known example of charged polymers, and they are characterized by containing bound ionic groups of either positive or negative charge. At low concentration and in absence of added electrolytes, the chains of linear polyelectrolytes in aqueous solutions are extended because of coulombic repulsion. They typically respond to added electrolytes by a decreased viscosity as the salt concentration is increased. This is due to charge screening which lowers the electrostatic repulsion between the ionic groups in the polymer. The large number of charged groups within a chain thus makes linear polyelectrolytes readily soluble in water, but they tend to adopt a contracted conformation and may eventually contract into a globular form and even precipitate if salt is added to the solution.

Polyampholytes constitute a special class of polyelectrolytes which contain both positive and negative groups, typically in separate monomer units. A subgroup of polyampholytes is made up by polybetaines, in which each monomer unit carries both a positive and a negative ion. Depending on the nature of the ionic groups, polybetaines may be divided into different types, such as polycarbobetaines, polyphosphobetaines, and polysulfobetaines, which are addressed here. The review by Kudaibergenov et al. is recommended for a more detailed summary of polymeric betaines.1
Zwitterionic polymers can be prepared by two basic routes, either by postmodification of a preformed polymer, or more commonly, by polymerization of zwitterionic monomers.

![Polyelectrolyte, Polyampholyte, Polyelectrolyte complex, Polybetaine](image)

**Figure 2.** Different types of ionic polymers.

Depending on the nature of the ionic groups of a polyzwitterion, the dual charge may persist throughout the entire pH range, resulting in a zero net charge of the polymer, or, if the polyzwitterion contains weak ionic groups, the polymer can acquire a net charge at certain pH values. Sulfobetaine polymers are characterized by the presence of a positive quaternary ammonium group and a negatively charged terminal sulfonate group, but may otherwise vary substantially in their structure. Figure 3 shows the general structure of sulfobetaine monomers. The work described in this thesis concerns polymers made from the methacrylate monomer 3-[2-(N-methacryloylethyl)-N,N-dimethylammonio] propane sulfonate, hereafter referred to as SPE. The aim was to develop new techniques for the controlled polymerization of SPE, both for the preparation of homopolymers in solution and by graft polymerization onto various chromatographic supports, and to study the interactions of SPE polymers with biomolecules in order to obtain a more detailed understanding of how the retention mechanism works.
3.1 Solution properties of sulfobetaine polymers

As previously mentioned, sulfobetaine polymers, as well as other zwitterionic polymers, have unique properties, manifested mainly in their solution behavior. In contrast to polyelectrolytes, polysulfobetaines are insoluble in water, but may be dissolved by addition of an electrolyte, a characteristic usually described as “antipolyelectrolytic behavior”2-7. The reason for the pronounced water-insolubility is the strong intra- and intermolecular quadrupolar association between the oppositely charged ionic groups of the polymer. The monomer units in the polymer tend to adopt a cyclic conformation to form an ion-pair, by association of the anionic tail group with the positively charged ammonium group. In addition there might also be intrachain associations between neighboring ionic groups within a single polymer chain, and interchain ionic bonds between individual polymer chains. The presence of these strong ionic bonds makes the polymer adopt a collapsed conformation that is inaccessible to water molecules, and it thus precipitates in water. By the addition of an electrolyte, for example regular NaCl, the polymer can however easily be dissolved. The ions from the salt shield the ionic groups in the polymer, which causes the strong ionic bonds to dissociate, and thereby disrupts the dense polymer network, so that the polymer chains may expand into the water phase. The dissolution of polymer chains is associated with a salt dependent
increase in viscosity of the solution. The ability of a salt to promote solvation of sulfobetaine polymers depends on the properties of the individual ions, and follows the order described in the well-known Hofmeister series\(^4\,6-9\).

The Hofmeister series was originally used to classify ions according to their ability to stabilize the structure of proteins in aqueous solution, but is now generally considered as a ranking of ions according to their effect on water structure. Chaotropic ions (water structure breaking ions), which are usually large ions with an evenly distributed and hence low charge density, interact better with the ammonium groups of sulfobetaine polymers and are thus required in a lower concentration in order to dissolve the polymer compared to kosmotropic ions (water structure breaking agents), which are typically small ions or ions with oriented charge, or ions of higher charge. The behavior of sulfobetaine polymers may also be explained in terms of the Pearson principle, in which acids and bases are described as either soft (large radius, low charge density) or hard (small, high charge density)\(^10\). According to Pearson’s theory, soft acids (cations) preferably bind to soft bases (anions) and vice versa, which corresponds well with the behavior observed for sulfobetaine polymers.

The antipolyelectrolyte effect of polysulfobetaines, manifest as a chain expansion upon addition of salt, has made them interesting in many application areas, for example as additives for drag reduction, enhanced oil recovery, and recovery of transition metal ions. Their potential in the biotechnology field, where their stimuli-responsive and biocompatible properties are exploited more specifically, has somewhat surprisingly only started to receive increased attention during the last two decades.

### 3.2 Biocompatible and protein resistant properties of sulfobetaine polymers

Phosphobetaines have been known for their biocompatible properties for a long time, a feature that has been mainly ascribed to their resemblance to the phospholipids in mammalian cell membranes\(^11-16\). Sulfobetaines have only recently been acknowledged for their ability to withstand protein adsorption, but several studies show that their protein resistant
properties are comparable to those of phosphobetaines. Non-detergent sulfobetaines have been shown to prevent aggregation of proteins in solution by acting as folding helpers \(^{17}\), and in 2001, Holmlin \textit{et al.} investigated the protein resistant properties of self assembled monolayers (SAMs) made from thiols functionalized with either sulfobetaine groups or 2-methacryloyloxyethyl phosphorylcholine (MPC)\(^{18}\). The adsorption of protein onto the sulfobetaine type SAMs decreased with increasing ionic strength of the aqueous buffer, and they were more efficient in resisting protein adsorption than the MPC SAM. Other authors have described ATRP mediated grafting of surfaces with both SPE\(^{19},^{20}\) and its analogue SPP ((3-(N,N-dimethyl-N-methacryloyl-amidopropyl) ammonium propane sulfonate)\(^{21}\), and the resulting polymer surfaces proved to have an ability to resist nonspecific protein adsorption comparable to the best known systems. Kitano modified gold surfaces with polymers of SPP, MPC, and \(N,N\)-dimethyl-\(N\)-(1-carboxymethyl)-2-methacryloyloxyethyl ethanaminium inner salt (CMB) and found no significant protein adsorption\(^{22}\). The protein resistant properties of copolymers containing sulfobetaine moieties appear to be related to the molar proportion of zwitterionic units in the polymer\(^{23},^{24}\), but a comparison with phosphocholine type copolymers still indicate better results for the latter\(^{25}\).

Although the term biocompatibility has traditionally been used in medicine, for example in the field of surgical implants and tissue engineering, the use of biocompatible materials outside of the human body is equally important. The growing interest in biosensors in analytical chemistry is only one example of an application where biocompatibility and protein resistant properties are of great importance to the end result. A continuous development of new and more specifically designed polymer materials would certainly improve the performance of many areas in biotechnology.

### 3.3 Sulfobetaines in chromatography

The separation of analytes in liquid chromatography occurs as a result of the combined actions of interaction with the stationary phase, and solvation in the liquid mobile phase. The large number of possible combinations of stationary and mobile phases enables the
chromatographer to finely adjust the separation parameters to fit a specific separation task, which makes liquid chromatography a very versatile technique. There are many sub-techniques in liquid chromatography, of which reversed-phase (RP) and ion exchange chromatography (IEC) are the most widely used. In IEC, the stationary phase typically contains ionic groups, either positively or negatively charged, while the eluent contains a salt that provides counterions to the stationary phase. The separation of ions in a sample is accomplished by an exchange process, where retention is determined by the varying affinity of the analytes for the ionic stationary phase. As early as in 1951, shortly after the introduction of ion exchange resins, Stach presented an attempt to make zwitterionic resins that contained both anion- and cation-exchange groups in a single particle, but the material was not evaluated. Nowadays, the definition of true zwitterionic ion-exchange materials states that they should hold both positive and negative groups in a single particle, as opposed to the mixed bed columns in which anion- and cation-exchange resins are combined.

Even when this requirement is fulfilled, zwitterionic resins can be further divided into several types. Polyampholytes contain ionic groups throughout the entire volume of the material, whereas pellicular resins consist of a layer of charged molecules deposited onto or attached to a support particle. Ion exchange materials contain oppositely charged groups at the surface of the resin, either in separate entities or within a single molecule, as is the case with the sulfobetaine polymers discussed here. There is also a group of chromatographic materials intended for ion separations that are termed surface agglomerated resins, wherein the carrier particles are furnished with a thin layer of charged groups, which are used to electrostatically attract an ion exchange latex of the opposite charge. These materials are not intentionally zwitterionic but still carry both charges, although the charged groups of the attached latex are in large excess.

### 3.3.1 Examples of sulfobetaines in LC

Betaines were the first zwitterionic entities that were considered for use in ion-exchange materials, mainly for the separation of ions in solution. In 1981, Knox and Jurand presented the use of zwitterionic ion-pairing agents in reversed phase chromatography, but it was not until Hu
adsorbed zwitterionic surfactants onto an octadecyl silica (ODS) column that the possibilities of zwitterionic materials were more widely recognized. The technique of treating ODS columns with zwitterionic surfactants is designated electrostatic ion chromatography (EIC) and has been successfully used to separate inorganic and organic ions with pure water or weak aqueous solutions of electrolytes as eluents. The concept of physically adsorbed molecules poses, however, an obvious risk of column bleeding, which led to attempts to produce silica based phases with covalently attached zwitterionic molecules. In 2002, Jiang and Irgum presented incorporation of sulfobetaine functionality onto silica beads by surface initiated graft polymerization of SPE\(^{30}\). They also modified polymer beads to hold sulfobetaine moieties, both by step-wise modification of hydroxyl-terminated beads\(^{31,32}\), and by direct grafting of SPE onto particles with methacrylate functionality\(^ {30,32}\). The resulting materials were successfully used to simultaneously separate inorganic anions and cations, as well as proteins. The latter example, separation of proteins, is especially interesting because of the documented protein friendly properties of sulfobetaine materials. The separation and analysis of proteins in liquid chromatography often involves reversible adsorption of the protein onto the stationary phase, and this is associated with a high risk of denaturing the protein. It is often of interest to maintain the native protein conformation throughout the separation, as its conformation often differs markedly from the denatured form, and thus may affect retention of the sample. Sulfobetaine stationary phases have the potential of providing “soft” separations of proteins because of their tendency to resist protein adsorption at the same time as efficient separation based on ionic interaction takes place\(^ {33,34}\).

The retention mechanism of zwitterion chromatography (ZIC) is clearly different from conventional ion exchange chromatography. The elution order of both anions and cations in ZIC differ somewhat compared to IEC, and it is in some cases possible to elute ions using only pure water as the eluent. Even though the retention mechanism on zwitterionic stationary phases has not been fully elucidated, a combination of two major components appears to be responsible for the retention behavior\(^ {35}\). The first is electrostatic attraction of the analytes to the stationary phase, and the second involves hydrophobic attraction and water-structure induced pairing of ionic groups. The elution of anions follows the
increasing chaotropic character described in the Hofmeister series, which is associated with their interactions with the quaternary ammonium groups in the stationary phase. The extent of the electrostatic versus hydrophobic attraction forces in the column depends partly on the properties of the analyte ion, but also by the nature of the stationary phase. Experiments with stationary phases with varying charge separation have shown that the ion-exchange character of the stationary phase increased with a greater charge separation between the ionic groups \(^{36}\). For more extensive reviews of zwitterionic ion-exchange materials or general retention models in ion chromatography, the works by Nesterenko \(^{37}\) and Stålberg \(^{38}\) are recommended.

### 3.3.2 Telomers for modification of chromatographic supports

An alternative route to achieve covalently bonded zwitterionic stationary phases is to attach pre-formed telomer chains onto a chromatographic support. A telomer is defined as a polymer chain carrying a terminal functional group, which may then be used to covalently couple the chain to some appropriate handle on the resin. The SPE telomers prepared in Paper I are intended for direct coupling onto polymeric particles (“grafting to”), and experimental work within this area is currently ongoing.

The advantage of the “grafting to” technique is that the molecular weight and structure of the grafted chains can be controlled prior to the grafting, and that it presents a simple way to prepare materials with mixed mode character, by simultaneously attaching different chains to the surface, or by attaching telomers that are small block copolymers. A potential problem sometimes associated with “grafting to” is low surface coverage, especially if the grafted chains are long or contain sterically hindered groups. The “grafting from” approach, in which polymer is grown directly from the surface, was used in Paper II, and will be discussed later. The “grafting from” approach is typically associated with a decreased level of control over the size of the grafted chains, although recent developments in controlled polymerization techniques from surfaces have been made \(^{39-48}\).
3.4 Characterization of modified materials

The characterization of separation materials used in chromatographic columns is important for several reasons. From a theoretical point of view, knowledge about both the surface structure and the bulk material are essential when retention models are used to explain experimental observations. Careful characterization of the stationary phase is also a key feature in the development of new materials, and should therefore receive proper attention. Some techniques that are particularly valuable are elemental analysis and X-ray photoelectron spectroscopy (XPS), which can be used for the characterization of both the support material as well as the functionalized stationary phase material. While elemental analysis yields information about the total amount of elements in the material, XPS is a surface sensitive technique that can be used to assess the presence of various surface groups. It is useful in order to verify that functionalization or grafting of a surface has occurred, and may also be used as a tool to determine the composition of telomers covalently bound to the stationary phase. This is done by comparing the relative amounts different species found on the surface to the known relationship of functional groups within the polymer. Characterization of telomers will be discussed in more detail in Chapter 4.

In addition to the selectivity of a column provided by the chemical structure of the stationary phase, the porous properties of the resin are of paramount importance to the chromatographic performance of a column. Conventional HPLC columns are typically made from functionalized microporous particles, which are carefully packed into a column holder. In the case of particulate packings, the particle diameter and the particle size distribution determine, in combination with the ratio between the column and particle diameters, how well the particles can be packed into a column bed. This will affect the interstitial volume, and thus the efficiency, of the final column. The porous properties of the column are important both for the flow-through characteristics of the column and for the mass-transfer of analyte to/from the stationary phase. Large inter-particle pores will promote a fast flow through the column as the back pressure will be relatively low, but the mass transfer will however be less efficient as the analytes have to travel through a large volume of stagnant mobile phase before reaching the stationary phase surface. A
particle deviating substantially from the ideal hexagonal (cubic) dense packing will also be inferior due to a spatially uneven flow resistance.

Monolithic columns, in which the separation material consists of a single porous entity, typically in the shape of a rod, consist of a bicontinuous network of interlinked particles, separated by relatively large pervading pores. Such materials have found some use in applications requiring high throughput, for example in protein separations. Regardless of the format, characterization of the pore size distribution, surface area, and particle size of separation materials is done on a routine basis, using techniques such as nitrogen adsorption measurements (as in determination of the BET surface area), mercury intrusion porosimetry, or inverse size exclusion chromatography. Typically, there is a trade-off between column efficiency and flow-through properties which will have to be considered for a specific chromatographic application.

4. RAFT polymerization of SPE

A key goal in this thesis work was to prepare zwitterionic polymers that can be attached to the surface of carrier materials and thereby transform their separation properties into ZIC type selectivity. One of the most versatile and common polymerization techniques today is free radical polymerization of vinylic monomers. In this process, the polymerization starts when an initiator decomposes to form reactive radicals which add to the double bond of a monomer and thereby starts a chain polymerization reaction. Free radical polymerization suffers from a number of limitations however, such as a broad distribution of molecular weights, low monomer conversion, and non-linear kinetics. In the late twentieth century, several techniques for controlled, or so called “living”, free radical polymerization were developed, of which nitroxide mediated polymerization (NMP), atom transfer radical polymerization (ATRP), and reversible addition fragmentation chain transfer (RAFT) polymerization are the most important techniques used today. The use of controlled polymerization techniques has made it possible to prepare a wide range of polymers with well-defined composition, functionality, and topology, as well as molecular weight. Gradient copolymers, hyperbranched or star polymers, and graft polymers are just some...
examples of applied polymers that have been made by controlled free radical polymerization techniques. Below is a brief introduction to the most common techniques used today.

4.1 Atom Transfer Radical Polymerization (ATRP)

ATRP is based on the use of a catalyst system that includes a transition metal species, a complexing ligand, and a counterion that can bind to the metal center. The most common ATRP catalysts are based on a combination of copper halides and nitrogen-based ligands, as it has proven to be the most efficient reagent combination for a wide range of monomers. However, many other metal/chelator combinations capable of undergoing the reversible redox reaction required in the propagation step have also been successfully used. Control over the polymerization is obtained through the dynamic equilibrium between propagating radicals and dormant species that is established, and termination reactions are in principle avoided since the equilibrium is strongly shifted towards the dormant species. ATRP has been used to polymerize many monomers, and it has several advantages: End-group functionalization is simple, only catalytic amounts of the transition metal complexes are needed and perhaps most important, most initiators and catalysts are commercially available. However, removal of the transition metal complex from the final polymer is often necessary.

4.2 Nitroxide mediated polymerization (NMP)

NMP is the most common example of a system that utilizes stable free radicals (SFRs) to obtain control over a free radical polymerization. Both ATRP and NMP involve a dynamic equilibrium between dormant and propagating radicals, but in NMP the thermal cleavage of the dormant species occurs without the use of a catalyst. The first example of NMP was the use of TEMPO (2,2,6,6-tetramethyl-1-piperidinyl-N-oxyl) for the polymerization of styrene. NMP can be used with many types of monomers, but the high temperatures generally required limit its use mainly to systems based on high boiling organic solvents. It has been shown to be very difficult to obtain controlled polymerization of disubstituted alkenes, and it is also complicated to introduce end-group functionality to the polymers.
4.3 Reversible Addition Fragmentation Chain Transfer (RAFT) Polymerization

The RAFT polymerization mechanism is different from both ATRP and NMP in that it does not involve the persistent radical effect. Instead of the propagating radicals being reversibly trapped by a stable free radical or by the redox cycling of an organometallic complex, RAFT operates through a degenerative transfer process, where a series of addition/fragmentation reactions with a thiocarbonylthio compound (RSC(=S)Z) acting as a chain transfer agent (CTA) results in an equilibrium between active and dormant chains. Since it was first introduced in 1998 by the Rizzardo group\textsuperscript{55}, RAFT polymerization has gone through substantial evolution and is now one of the most popular techniques for controlled polymerization. The number of papers has grown steadily each year since 1998, and only in 2007, 334 papers were published on the subject\textsuperscript{b}. One of the reasons for the popularity of RAFT polymerization is its versatility, both in terms of monomer and solvent choice, as well as experimental conditions. Opposite to ATRP, which typically requires quite stringent reaction conditions, and NMP, which has to be carried out at relatively high temperatures, RAFT can be performed in many solvents, including water, under a wide range of polymerization conditions (bulk, solution, suspension, emulsion, etc.) and with a wide range of monomers\textsuperscript{56}. An important factor behind the success of RAFT is the possibility to combine different R- and Z-groups (cf. Figure 4) in order to find optimal CTAs for a large number of monomers. Most RAFT polymerizations are performed with dithiobenzoate CTAs, in which the Z-group is a phenyl moiety, as they have shown to provide particularly good control over the polymerization process\textsuperscript{57}.

4.3.1 General mechanism of dithiobenzoate mediated RAFT

RAFT polymerizations resemble conventional free radical polymerizations in terms of kinetics and polymerization rates, and only require the addition of a thiocarbonylthio compound (RSC(=S)Z to the polymerization mixture to act as a CTA. The general mechanism of RAFT polymerization is shown in Figure 4. The chain transfer agents

\textsuperscript{b} Based on a search on ISI Web of Science on May 18, 2008.
contain a stabilizing Z-group and a reactive R-group. Primary radicals are generated from a conventional free radical initiator, either by thermal decomposition or ionizing radiation. The primary radicals can either add directly to the RAFT agent to form an intermediate RAFT radical, or to monomer in the solution to form short oligomeric macroradicals ($P_i^·$), which may then add to the RAFT agent. In both cases, the end result is that the R-group of the RAFT agent is fragmented off to yield reactive carbon radicals, from which new polymer chains are initiated. Ideally, all polymer chains in a RAFT polymerization are formed by initiation by the R-group radicals, and the degree of polymerization is hence determined by the ratio of monomer to RAFT agent and not by the concentration of the initiator.

Radical generation  

Initiator $\rightarrow 2 I^·$

Pre-equilibrium

Main equilibrium

Figure 4. General mechanism of RAFT polymerization.

However, there is always a small fraction of chains that originate from direct initiation by the primary initiator radicals, but the contribution from these chains compared to the overall polymerization yield is small and can often be neglected. The theoretical molecular weight at a certain monomer conversion is thus given by
\[ M_{n,\text{theory}} = \frac{[M]_0 \times M_{w,\text{monomer}} \times \rho}{[\text{CTA}]_0} + M_{w,\text{CTA}} \]

where \([M]_0\) is the initial monomer concentration, \(M_{w,\text{monomer}}\) is the molecular weight of the monomer, \(\rho\) is the fractional monomer conversion, \([\text{CTA}]_0\) is the initial concentration of RAFT agent, and \(M_{w,\text{CTA}}\) is the molecular weight of the RAFT agent.

A RAFT polymerization can be described in terms of a number of addition/fragmentation reactions that occur throughout the reaction. When the conventional radical initiator is cleaved by heat or ionizing radiation to yield radicals, these (or any propagating chains that may have formed) add to the sulfur-carbon bond of the RAFT agent, forming an intermediate RAFT radical (IR\(^-\)). The intermediate radical can undergo two alternative fragmentation reactions, either fragmentation of the original primary radical or polymer chain, yielding back the original molecules, or loss of the R-group of the RAFT agent, yielding a reactive R-group radical (R\(^-\)) and a dormant polymeric RAFT agent. The process in which the initial RAFT agent is transformed into polymeric RAFT agent, releasing secondary R-group radicals into the solution, is called the pre-equilibrium (sometimes also referred to as the initialization period). In the main equilibrium, similar addition and fragmentation reactions control the transition of polymer chains to and from the RAFT agent until most of the monomer has been consumed, ideally leading to a well-controlled polymer of low polydispersity.

As in conventional free radical polymerization, a steady-state concentration of radicals is established in the solution, but in RAFT polymerization this occurs through the equilibrium between dormant and active chains rather than through initiation and termination reactions. However, a small supply of initiator-derived radicals is also produced throughout RAFT polymerizations. At any moment, only a minor fraction of the polymer chains are in the active, propagating state, and growth of the polymers should only occur by insertion of a few monomer units at a time before the radical adds back to another CTA. The degree of polymerization in RAFT, and thus the molecular weight of the final polymers, is controlled by the molar ratio of monomer to CTA. The ratio between the CTA and the initiator needs to be
considered as well, as it affects the polymerization rate. The [CTA]/[I] ratio should be kept sufficiently high to ensure that the majority of all polymer chains will be initiated by the R-group radicals rather than by the conventional initiator, while still maintaining a reasonable initiation rate. Typically, the CTA concentration is about 10 times higher than the initiator concentration.

4.3.2 Rate retardation

A common feature in dithiobenzoate mediated RAFT polymerization is the decrease in polymerization rate compared to normal free radical polymerization that is often observed, both in terms of a delayed onset of the polymerization, commonly denoted an inhibition period, as well as a slower polymerization rate in general. Rate retardation is believed to be a combination of slow fragmentation of the macro-RAFT adducts, leading to a buildup of dormant polymer chains, and irreversible (and/or reversible) intermediate radical termination, which also would lead to a decreased number of growing polymer chains.

Both rate retardation and inhibition typically become more prominent as the concentration of the CTA, or its stability, increases. This has been shown both in experimental work and in model simulations\textsuperscript{58}, but the mechanisms behind this non-ideal kinetic behavior have been under debate during the past years\textsuperscript{57, 59, 60}. Even if the different models that have been developed differ slightly, they yield similar results when used to predict polymerization kinetics and polymer molecular weights.

According to the proposed models, an increase in dithiobenzoate CTA concentration typically leads to more pronounced rate retardation, which agrees well with experimental observations from numerous RAFT polymerizations, as it shifts the RAFT equilibrium towards the dormant species rather than towards propagating radicals. The addition and fragmentation reactions that occur in RAFT polymerizations are controlled by several rate constants, which determine the relative concentration of the different radicals and dormant species in solution. If the fragmentation rate constant of a CTA-polymer adduct is high, intermediate radicals may accumulate in solution, which results in retarded polymerization kinetics. Both the concentration and structure of the CTA used in the polymerization are furthermore of importance. A
CTA with an easily eliminated R-group would fragment at a higher rate, thus reducing the risk of rate retardation. A stabilization of the intermediate radical causes the fragmentation rate constant to increase, which reduces the possibility for fragmentation to occur. The pronounced rate retardation observed in dithiobenzoate mediated polymerizations can thus be explained by the strong stabilizing effect of the phenyl Z-group. Several resonance structures are formed as a result of the delocalization of the electron in the aromatic system, making the IR very stable, as shown in Figure 5.

![Figure 5. Resonance structures of the intermediate radical in dithiobenzoate mediated RAFT polymerization.](image)

The rate limiting step during the inhibition period appears to be the initialization process in which RAFT agent adds to monomer. McLeary found that the RAFT agent must be fully converted into single monomer adducts before additional propagation of the polymer chains can occur, thereby causing the commonly observed inhibition or initialization period\(^\text{61}\).

The success of a RAFT polymerization is to a large extent associated with the rate constants of both the pre-equilibrium and the main equilibrium, which in turn depend on the combination of monomer with a suitable CTA. In order to achieve a highly controlled polymerization, the rate of transfer to the CTA should be fast compared to the rate of propagation, so that at most a few monomer units are incorporated into the propagating chain in each fragmentation-chain transfer cycle.

### 4.3.3 RAFT in aqueous systems

One of the great advantages of RAFT is its versatility in the preparation of water-soluble polymers, both in fully aqueous systems or in organic
solvents. Controlled homogeneous aqueous polymerization of hydrophilic monomers has been attempted both with ATRP and NMP, but the limitations in terms of monomer choice and polymerization conditions make the use of these techniques rather limited. RAFT polymerization has on the other hand proven to be highly versatile in aqueous polymerization, as demonstrated by the wide range of hydrophilic monomers, including various anionic, cationic, and zwitterionic monomers, that have been successfully polymerized by RAFT.

Performing RAFT polymerizations in aqueous environment is, however, associated with some practical limitations. Most RAFT agents are susceptible to hydrolysis to a certain degree, and care must be taken that the RAFT agents are not degraded during the polymerization, as this will result in a broadened polydispersity index (PDI) as well as loss of the chain end functionality of the polymer. The first examples of aqueous RAFT polymerizations were performed using water-soluble dithioester and thioicarbonate CTAs that had a limited stability in water, and the need to develop new CTAs with improved water stability was soon acknowledged. Baussard et al. and Mertoglu et al. have reported the synthesis of several new CTAs suitable for aqueous RAFT polymerizations. Although some of the newly developed CTAs have shown increased stability compared to those used in the initial aqueous polymerizations, most water-soluble RAFT agents are still sensitive to alkaline pH and elevated temperatures. The ability to perform aqueous RAFT polymerizations at ambient temperature would thus be beneficial both in terms of RAFT agent stability, but also in applications utilizing heat sensitive monomers. In this thesis, UV-initiated polymerization RAFT polymerization in a fully aqueous environment was investigated, particularly with focus on the zwitterionic monomer SPE.

4.4 Controlled polymerization of SPE in solution

The zwitterionic monomer SPE has proven to be inherently difficult to polymerize in a controlled fashion, both because it polymerizes rapidly, and because of the limited solubility of the polymer in various solvents, including water. Monomeric SPE is readily water soluble, but because of the strong bonds that are formed between the ionic groups in the
polymer chains (cf. Section 3.1), it is virtually insoluble in water as well as in all organic solvents except for 2,2,2-trifluoroethanol. In order to keep the polymer chains in an expanded state during the polymerization, so that they are easily accessible to propagation of monomer, the polymerization should be carried out in aqueous salt solutions. In 2002, Donovan et al. reported controlled polymerization of homopolymers based on SPE and two other zwitterionic monomers using RAFT polymerization in aqueous solution. They were able to produce SPE polymers with a relatively high molecular weight, but noted that SPE polymerized considerably faster than the two acrylamido and styrenic analogues also investigated. The SPE polymers synthesized in this thesis are intended to be used as stationary phases in liquid chromatography, and therefore we wanted to produce considerably shorter polymers chains, preferably in the range between 5 to 50 monomer units.

4.4.1 Iniferter mediated polymerization of SPE

The initial attempts to synthesize SPE oligomers of controlled size were performed using UV-initiated iniferter-mediated polymerization. This polymerization technique, introduced by Otsu, utilizes compounds that acts as initiators, transfer agents, and terminators, typically molecules containing a C=S bond. After cleavage of the iniferter by heat or radiation to produce radicals, the polymerization process is believed to occur through repeated steps of propagation and subsequent recombination with (stable) radicals from the iniferter, so that an equilibrium between dormant and active chains is established, ultimately leading to a controlled polymerization. In addition to this, the use of an iniferter results in the incorporation of a functional disulfide or thiol group at the polymer end, which may be advantageous in grafting reactions. Zwitterionic monomers have been polymerized with the iniferter technique, for example SPP, which is the acrylamido analogue to SPE. Although an iniferter moiety was apparently incorporated in the polymer, as judged by the successful immobilization of the telomers on a gold surface, the degree of control over the polymerization was not demonstrated.

In the case of SPE, the iniferter polymerizations were made with two different iniferter molecules, 4-(N,N-diethylthiocarbamoyl-methyl)-benzoic acid-cystamine conjugate (Cys-BDC), which was synthesized in
our lab, and isopropylxanthic disulfide, which is commercially available. None of these are water-soluble, and the polymerizations were therefore performed in solvent mixtures containing varying amounts of methanol, THF, water, and NMP (preliminary experiments). Since the SPE polymer is insoluble in all of these solvents, LiClO$_4$ was added to the polymerization mixtures to facilitate dissolution of the formed polymers. It was, however, not possible to achieve a controlled polymerization of SPE under these conditions. In all cases, a gel-like, sticky substance was formed at the bottom of the vessel, indicating the formation of long polymer chains. The difficulties to achieve controlled polymerization of SPE with the iniferter were probably a combination of several factors. An important issue was probably the poor solubility of the SPE polymer in the solvent mixtures required to dissolve the iniferter. Polymer precipitates were formed within seconds after the polymerization mixtures were subjected to the UV-radiation, and propagating (or terminated) polymer chains were thus rapidly transported away from the solution, which of course makes interaction of the propagating radical with the iniferter highly unlikely.

4.4.2 Aqueous polymerization of SPE via RAFT (Paper I)

In order to achieve a controlled polymerization of SPE, it seemed necessary to perform the reaction in a solvent capable of dissolving both the SPE monomer and the polymer. This can basically only be accomplished in aqueous salt solutions, which rapidly narrowed down the number of available polymerization techniques. The demonstrated ability of water soluble RAFT agents to yield controlled polymerization in water made this technique seem like a suitable choice for the polymerization of SPE. Although RAFT polymerization has previously been used for the polymerization of zwitterionic monomers\textsuperscript{66, 68, 69}, only polymers with higher molecular weights were synthesized. Actually, examples of RAFT polymerizations targeting very low molecular weight are rare, and the effect of the high CTA concentrations that are required have only been studied in a few papers\textsuperscript{59, 70}.

4.4.2.1 Choice and synthesis of chain transfer agents

The water soluble RAFT agent sodium 2-(2-thiobenzoylsulfanyl propionylamino) ethanesulfonate (STPE) was chosen for the
polymerization of SPE. The success of a RAFT polymerization largely depends on the combination of monomer with a suitable RAFT agent, and STPE was chosen because of the similar structure of the ethanesulfonate end group of STPE with the sulfonate group of the SPE monomer. STPE has shown good long-term stability towards hydrolysis in water, both at elevated temperatures and at acidic pH values, but is slightly sensitive to alkaline pH\textsuperscript{63, 64}. The first attempt so synthesize a water-soluble chain transfer agent however aimed at the similar compound sodium 2-(2-methyl-2-thiobenzoylsulfanyl propionylamino) ethanesulfonate, which was believed to be a better choice for methacrylate monomers such as SPE\textsuperscript{64}. This CTA differs from STPE only by an additional methyl group at the carbon adjacent to the dithiobenzoate group, but proved to be considerably more difficult to synthesize, as the high temperature necessary to achieve coupling of the tertiary alkyl halide resulted in decomposition of the dithiobenzoic acid.

![1H NMR spectrum of the chain transfer agent sodium 2-(2-thiobenzoysulfanyl propionylamino) ethanesulfonate (STPE) used in the RAFT polymerization of SPE.](image)

Even after several attempts to synthesize this molecule, the reaction yield was only about 10\%, and STPE was chosen instead. The \textsuperscript{1}H NMR spectrum of STPE is shown in Figure 6. A small amount of thioester by-product was formed in the synthesis, which resulted in additional peaks in the \textsuperscript{1}H NMR spectrum at 1.3, 1.5, 3.3, and 4.3 ppm. The presence of
thioester in the RAFT agent has been shown not to affect the performance of STPE as a RAFT agent, and the product was therefore used without further purification.

4.4.2.2 UV-initiated polymerization of SPE

In order to achieve a fast and efficient polymerization, UV-initiated RAFT polymerization in water was attempted. Although the stability of the water soluble CTAs used today is often satisfactory, the use of mild polymerization conditions is advantageous, for example when temperature-sensitive monomers are used, or during the preparation of polymer-protein conjugates. UV-initiated polymerization also has other benefits, for example a high efficiency, and the possibility to quickly stop the polymerization reaction by simply turning off the light. The first successful RAFT polymerization at low temperature using UV-initiated photopolymerization was performed by Quinn et al., who polymerized styrene with a dithiobenzoate CTA at 365 nm. The molecular weight distribution broadened considerably at long irradiation times, which they concluded was the result of decomposition of the CTA moieties at the polymer ends. Lu et al. polymerized methyl acrylate and methyl methacrylate in the presence of trithiocarbonates or dithioesters as CTAs, and also experienced that the control over polymerization was lost after some time. They moved on to investigate the effect of radiation wavelength on the RAFT process, and found that the CTAs were significantly degraded at wavelengths below their characteristic absorption wavelength. The risk of CTA degradation can be minimized by using an appropriate wavelength, for example by cutting off the short-wave UV radiation by using a filter.

In paper I, SPE was polymerized at room temperature using UV-initiated RAFT polymerization in water. The water-soluble azo compound 2,2'-azobis(2-amidinopropane) dihydrochloride, commonly used in thermal polymerizations, was used as a photoinitiator together with sodium 2-(2-thiobenzoylsulfanyl propionylamino) ethanesulfonate (STPE) as the CTA. The study showed some interesting kinetic results that deviated from typical RAFT behavior. SPE was polymerized rapidly, and a monomer conversion of over 90% was achieved in about 90 minutes. There was no inhibition period, which is typically observed in most dithiobenzoate mediated RAFT polymerizations. Although the
polymerization was very fast, the polymerization appeared to proceed at a constant rate up to high monomer conversions. Figure 7A shows the plot of \( \ln([M]_0/[M]_t) \) against polymerization time for three different targeted molecular weights. There was a nearly linear increase of \( \ln([M]_0/[M]_t) \) versus time for the RAFT polymerizations, which indicated that monomer conversion occurred at a relatively constant rate throughout the polymerizations. The polymerization rate decreased when the monomer conversion exceeded 90\%, which corresponds to a \( \ln([M]_0/[M]_t) \) value of 2.3.

**Figure 7.** Plots of \( \ln([M]_0/[M]_t) \) versus polymerization time for UV-initiated (A) and thermal (B) RAFT polymerizations of SPE.

The \([M]/[CTA]\) ratio had no effect on the polymerization rate, at least not in the initial stages of the polymerization. A higher concentration of CTA in the polymerization mixture is generally expected to result in more rate retardation, but no such effect was observed here. Instead, the slightly increased polymerization rate that was observed as the \([M]/[CTA]\) ratio decreased was probably a result of a higher initiator concentration. As the \([CTA]/[I]\) ratio affects the number of initiator derived radicals and hence the initial polymerization rate, this ratio was kept constant in all polymerizations. However, the total concentration of radicals will of course increase as the CTA concentration increases, which might explain why the polymerization rate actually increased slightly. A similar observation was recently made by Pichot *et al.*, who studied RAFT polymerization of several acrylamide derivatives\textsuperscript{75,76}. The
high CTA concentrations used for the preparation of low weight polymers did not affect the polymerization rate as long as the [CTA]/[initiator] ratio remained constant. The authors suggested that the rate retardation induced by the increased CTA concentration was perfectly balanced by the accelerated polymerization rate resulting from a higher initiator concentration.

It thus seems likely that the similar polymerization rates that were observed in the UV-initiated polymerization of SPE as the CTA concentration was increased are the result of an increase in the initiator concentrations. This apparently compensates for the effects of rate retardation, and even partly overshadows them. Another possible effect of a higher initiator concentration is a faster transformation of RAFT agent into macro-CTA molecules, which would decrease the duration of the inhibition period.

### 4.4.2.3 Thermal polymerization of SPE

The results from the UV-initiated polymerization of SPE were compared with a number of reference polymerizations conducted by thermal initiation at 65 °C in an oven. Figure 7B shows the results from the thermal polymerizations, including data from a batch with a [M]/[CTA] ratio of 170 (target molecular weight 49 000), which was added to expand the concentration range of the CTA. The major difference seen between the UV- and thermally initiated polymerizations was the rate of monomer conversion at the beginning of the reactions. While polymerization started almost immediately in the UV-initiated polymerizations, there appeared to be an inhibition period of about 15 minutes in the thermal polymerizations. This agrees with the results found by Donovan, who noticed an inhibition period of about 10 minutes in RAFT polymerization of SPE conducted with sodium 4-cyanopentanoic acid dithiobenzoate as the RAFT CTA.

The monomer conversion was significantly slower in the initial stages of the thermal polymerizations. A comparison of the batches with [M]/[CTA]=50 showed that after 15 minutes, the conversion was already 30 % in the UV initiated polymerizations, while no monomer conversion had occurred in the thermal polymerization. After 30 minutes, the conversion was still only 7 % in the thermal polymerization, but the polymerization rate then increased and the final conversion was
comparable to that of the UV-initiated batch. Part of the inhibition period observed in the thermal polymerization is most likely caused by a non-established radical equilibrium, as the initiator decomposition rate is probably lower in the beginning of the reaction until the polymerization solution has been heated. The absence of inhibition in the UV-initiated polymerizations is a sign of rapid establishment of the pre-equilibrium. Once the polymerization mixture is exposed to UV radiation, primary radicals should be released within seconds and initiate the polymerization. Both rate retardation and inhibition periods are common in dithiobenzoate mediated RAFT polymerizations, and typically become more pronounced as the CTA concentration increases. Although we did not observe any lengthening of the inhibition as more CTA was used in the thermal polymerizations, monomer conversion started earlier and proceeded at a higher rate when no CTA was added to the solution, which indicates that the CTA caused a short inhibition period in the thermal polymerization.

4.4.2.4 Polymerization of SPE without CTA

The linear kinetics observed in the RAFT polymerizations indicates that the polymerization was under control, even if it probably does not meet all the criteria for an ideal “living polymerization”. However, since the kinetic behavior of the RAFT polymerization was quite unexpected, in terms of the short inhibition period and the relatively fast polymerization rate even at high CTA concentrations, SPE was also polymerized without CTA in order to compare the kinetics with those of a conventional free radical polymerization. A polymerization solution identical to the RAFT polymerization with [M]/[CTA]=50, but without any CTA, was prepared and polymerized both by UV light and by thermal polymerization.

The difference in the kinetics between the RAFT-mediated polymerization and the conventional free radical polymerization is clearly seen in Figure 8. The initial polymerization rate for the reference batch was about twice as high as for the RAFT polymerizations, which resulted in 70% monomer conversion after only 15 minutes. After that, the rate decreased and polymerization finally ceased at 95% monomer conversion.
This is still a very high conversion for a free radical polymerization, where termination reactions between the propagating chains usually result in moderate conversions. The UV-initiated RAFT polymerization followed pseudo-first order kinetics, which is typically observed in RAFT polymerizations.

The polymerization rate was clearly slower when CTA was present in the polymerization solution compared to the conventional free radical polymerization. Although not shown in the figure above, monomer conversion continued for some time in the RAFT polymerization, while it ceased already after one hour in the conventional polymerization.

4.4.7 Criteria for controlled free radical polymerization

A key issue in the evaluation of RAFT polymerization, as in all free radical polymerization techniques aspiring to demonstrate living behavior, is whether the polymerization fulfills the requirements of a controlled polymerization. These criteria have been discussed in the literature, but the following principles apply to most systems. In an ideal “living”/controlled polymerization, termination and chain transfer reactions are absent and the polymers thus grow at a constant rate. As a result, the monomer should be fully consumed and the evolution of
molecular weight should increase linearly with monomer conversion. Initiation should be fast and occur simultaneously, and the number of propagating chains should thus remain constant throughout the polymerization, resulting in a low PDI. Another important criterion for a controlled polymerization is the possibility to control the final molecular weight of the polymers via reaction stoichiometry. It should be possible to prepare block copolymers by addition of a second monomer, and the integrity of the polymer chain ends must hence be maintained throughout the polymerization, also after propagation has ceased. These criteria of course apply to an ideal controlled polymerization, and a polymerization must not necessarily obey all of them in order to be regarded as controlled. The degree of control obtained in a polymerization can vary from relatively high to being essentially uncontrolled depending on the actual conditions. For most practical applications, the goal is to obtain a polymer with a pre-determined molecular weight and a low polydispersity, and therefore these characteristics are highly relevant.

A plot of $\ln([M]_0/[M]_t)$ against polymerization time is included in most papers describing RAFT polymerization in order to show pseudo first-order kinetics of the polymerization, and thus demonstrate that the system is under control. As pointed out by Lowe and McCormick, a linear pseudo first-order kinetic plot is not necessarily an absolute proof of a controlled polymerization, but can be observed in other free radical polymerizations that are under steady-state conditions.$^{62}$ If the plot is linear up to high conversions this is however indicative that autoacceleration, also known as the Trommsdorff effect is absent. Autoacceleration is often observed in free radical polymerizations performed in bulk or concentrated monomer solutions. It is characterized as the increase in polymerization rate that occurs after a certain monomer conversion when termination reactions becomes less frequent due to the reduced mobility of the viscous polymer chains, while the propagation rate remains unchanged.$^{77}$ In living polymerizations such as RAFT, autoacceleration is not observed since termination reactions (ideally) do not occur and polymerization thus proceeds at approximately the same rate throughout the whole polymerization.
4.5 Characterization of SPE telomers prepared by RAFT polymerization (Paper I)

Evaluation of a RAFT polymerization is closely associated with the characterization of the resulting polymers, both at the end of the polymerization, and during the polymerization in order to study the kinetics of the reaction. The choice of suitable characterization techniques for a certain polymer is dictated by the properties of the polymer in question, and may be associated with practical problems. SPE polymers have unique properties that make them particularly difficult to characterize, and some of these will be addressed in the sections below.

4.5.1 Size exclusion chromatography

Size exclusion chromatography (SEC) is a frequently used technique in polymer analysis in which polymers are separated according to their hydrodynamic volume. A SEC column contains a porous stationary phase where the elution volume is not determined by retentive forces between the analyte molecules and the stationary phase, but is based entirely on the size-dependent exclusion of an analyte from a certain fraction of the eluent contained inside the well-defined pore space of the stationary phase. Small molecules can enter a larger fraction of these pores, and thus spend a longer time traveling through the column, whereas larger molecules are partially or completely excluded from the pores, and elute in a shorter time. Above a certain molecular weight cut-off, the molecules are excluded from the entire porous structure and elute at a volume corresponding to the interstitial volume of the column plus the dead volume of the system.

Depending on the nature of the mobile phase, SEC is traditionally divided into two major application areas. When organic solvents are used as the mobile phase, which is common in polymer analysis, the term gel permeation chromatography (GPC) is used, whereas the term gel filtration chromatography (GFC) is frequently used in biochemistry and other fields where water is the mobile phase. In this thesis the term ASEC will be used to describe aqueous size exclusion chromatography. Although SEC is commonly used for molecular weight determination of
polymers, it is a relative technique that separates analytes on the basis of hydrodynamic radius and not molecular weight. The relationship between hydrodynamic radius and molar mass is not linear, and in addition it also depends on the chemical structure of the polymer and on the solvent used as eluent. Calibration with polymer standards of known molecular weight, preferably with a composition identical to the analyte, is thus necessary unless a detector capable of determining the molar mass is used. However, since the response of such detectors depends both on the concentration and the molecular weight of a particular polymer fraction, a concentration sensitive detector such as a UV-VIS or a refractive index (RI) detector is commonly required as well.

4.5.1.2 ASEC in RAFT polymerization of SPE

In the preparation of SPE polymers described above, ASEC was mainly used as a means to study the kinetics of the polymerizations by following the monomer conversion with time. Attempts were also made to use ASEC for the characterization of the formed polymers, but the molecular weights of the polymers could not be determined. The failure to obtain molecular weight data from the ASEC experiments can partly be ascribed to the lack of an appropriate calibration standard. SPE polymers are quite unique, and the use of commercially available polymer standards, such as polyethylene oxide, is possible but unquestionably yields biased results. There were also problems with the resolution of the SEC column used in the experiments. The difference in elution volume between the different polymer samples was relatively small and therefore only a relative estimation of the size of the polymers could be made. SEC analysis of charged polymers may be associated with possible formation of aggregates in the column, which has been noted previously.

4.5.2 Mass spectrometry

The quite limited resolution of SEC has led to exploitation of other techniques for molecular weight determination of polymers. Today, the mass spectrometry techniques are gaining a lot of interest. Matrix assisted laser desorption/ionization time of flight spectrometry (MALDI-TOF) is commonly used in polymer analysis, and it enables both determination of molecular weight distribution, and end group
Electrospray ionization mass spectrometry (ESI-MS) has also gained increased interest in the analysis of synthetic polymers, particularly in the case of multiply charged polymers. The techniques for analyzing polymer with mass spectrometry have developed substantially in recent years, but the data is often complex and requires careful interpretation and data treatment before information on the molecular weight distribution can be extracted.

Matrix assisted laser desorption/ionization time of flight spectrometry (MALDI-TOF) was initially attempted as a means to determine the molecular weight distribution of the synthesized SPE polymers, but the polymers proved extremely difficult to ionize. Despite extensive efforts in finding a suitable water soluble matrix, no signals were obtained. The difficulties in ionizing the zwitterionic SPE polymers can most likely be explained by its unique structure. Although the polymer contains many ionic groups, the net charge is zero. In addition, there is probably a strong tendency towards formation of ionic bonds as water is evaporated from the polymer-matrix solution. The unusual stability of zwitterionic polymers against ionization probably explains why we could not find any publications describing MS analysis of SPE homopolymers in the literature. A silica-based hybrid copolymer material containing zwitterionic moieties was recently prepared by Cheng and coworkers, who were successful in using MALDI-TOF for molecular weight characterization. In this case, the ionization of the sample was probably aided by the non-zwitterionic part of the polymer.

Contrary to MALDI, which is a soft ionization technique usually resulting in singly charged molecular ions, electrospray ionization (ESI) generates a distribution of multiply charged ions. In the case of polymers, the molecular weight distribution, which may be more or less wide, is associated with more complex mass spectra. We therefore investigated the possibility of using ESI-MS for the characterization of the SPE polymers. A challenge with electrospray is the use of volatile solvents, which must be able to fully solvate the polymer prior to injection in the electrospray ionization source. For the case of SPE polymers, a combination of ammonium acetate and methanol was used as the solvent, but no ionization of the polymer was observed. In an attempt to break the ionic structure of the polymer that we believed was
hampering the ionization, a small amount of sodium iodide was added to the polymer solutions. Iodide is a soft anion, particularly efficient in binding to the quaternary ammonium groups of the SPE polymer chains, and should therefore facilitate the ionization process. After addition of sodium iodide to the polymers, a few peak clusters appeared in the mass spectrum, all below an m/z ratio of 700. The typical distribution of peaks often seen in ESI-MS spectra was not observed, and each cluster only consisted of about 5-7 peaks. No further evaluation of the results was attempted.

4.5.3 Dynamic light scattering

Instead of an absolute molecular weight determination of the polymers, the hydrodynamic diameter of the polymers was measured with dynamic light scattering (DLS), using a Malvern Zetasizer Nano instrument. DLS provides information about the hydrodynamic diameter of a polymer chain in solution, rather than the molar mass. As in SEC, the use of a well-defined polymer standard may however allow for transformation of the size data into molecular weight. The conformation of a polymer chain, as any other macromolecule, is affected by the solution surrounding it. By measuring the hydrodynamic diameter of a polymer chain, the effect of ions and water associated with the polymer chain is taken into account. For chromatographic applications, knowledge about hydrodynamic size may actually be more valuable than about absolute polymer molecular weight, as the interaction between an analyte and, e.g., a chromatographic column with grafted tentacles depends on the quasi-solution properties of the grafted polymer chains, i.e., how the polymeric layer is swollen by the eluent and extends into the pore space.

Dynamic light scattering utilizes the Brownian motion of particles in solution, and the fact that particles of different size will move in solution by random walk at different velocities. Particles in a sample are illuminated with laser light and by detecting the fluctuations in the scattered light that occur as the particles move, it is possible to determine the size of the particles. The hydrodynamic diameter of the SPE polymers was plotted as a function of the [M]/[CTA] ratio, which is shown in Figure 9 below. There is a clear correlation between the targeted molecular weight and the hydrodynamic size, although the
values for the measured diameter differ somewhat depending on the measured variable.

![Graph](image)

**Figure 9.** Hydrodynamic diameter of SPE polymers as a function of [M]/[CTA] ratio.

For polymers, the number distribution is especially valuable, as it provides the relative number of particles of a certain diameter in a sample. Although there was a correlation between size and targeted molecular weight, we could not see any increase in size with monomer conversion for the individual batches. A RAFT polymerization is expected to proceed via a stepwise growth of the polymer chains, so that at any given time, all chains are of approximately the same length. It is difficult to determine whether this is the case in the polymerization of SPE. Due to the fast polymerization rate it was difficult to follow the monomer conversion at the beginning of the polymerization, but a relatively “constant” particle size was established already at low conversion. At this point, we do not have an explanation to this observation, but one possibility is that the apparent size of the polymer chains is not well correlated to the molecular weight. The conformation of zwitterionic polymers depends on the salt concentration in solution, but most studies have been made on high molecular weight polymers. The behavior of short chains has to our knowledge not been studied. Although the particle size measured by DSC appeared to be basically constant throughout the polymerization, the high overall monomer
conversion and the linear kinetics are good indicators of controlled polymerization.

4.5.4 Other techniques

Compositional analysis of polymers can be achieved by mass spectrometry, but other techniques may be employed. NMR, XPS, and elemental analysis are often used to determine the composition of polymers. An interesting technique is gradient polymer elution chromatography (GPEC) which has proven useful in the determination of copolymer composition. Another useful technique is FT-IR, which enables accurate identification of various functional groups such as carbonyl groups, esters, amines, etc. on the surface of a solid sample.

5. Modification of chromatographic supports with sulfoBetaine polymers

In liquid chromatography, surface modification of the resin has a great influence on the retention characteristics of a column. Traditionally, columns with charged stationary phases have been used in various modes of ion chromatography, but are also applicable in HILIC separations. Depending on the type of ions that are being separated, ion exchange columns carry either positively or negatively charged groups, resulting in anion or cation exchange columns respectively. Charged groups may be introduced on a surface in several ways. A convenient way to modify metal surfaces with alkyl chains is by using the self-assembled monolayer (SAM) technique, which works well also for charged molecules. The first part of this chapter describes some attempts to perform real-time studies of the behavior of surfaces functionalized with sulfoBetaine polymers in solution, while the second part focuses on zwitterionic polymers covalently attached to silica and polymer particles, and their application in chromatography.
5.1 Assessing the properties of surfaces with sulfobetaine polymer layers using QCM-D

The explanation behind the unique properties of surfaces functionalized with zwitterionic polymers is still not completely clear, but an important factor appears to be that the zwitterionic groups affect the properties of the water layer close to the surface\(^\text{17, 88, 89}\). In order to get a more detailed understanding of the structure and behavior of surfaces immobilized with zwitterionic polymers, as well as their interaction with ions and proteins, we explored various techniques for real-time studies of surfaces modified with zwitterionic polymers.

The quartz crystal microbalance (QCM) was introduced several decades ago and is used as a very sensitive device to measure the mass of various molecules adsorbed onto a surface. The QCM utilizes the relationship between the mass and resonance frequency of quartz crystals to adequately detect very small mass changes occurring on a surface. As the mass of the quartz crystal increases, for example by adsorption of a specific molecule, its resonance frequency decreases. By continuously monitoring the resonance frequency, minute mass changes of the crystal can thus be detected in real time. Initially, only gas-phase measurements were possible, but in the eighties, Nomura showed that the QCM could be operated in liquid as well\(^\text{90}\). Since then, the use of the QCM technique in liquid has undergone immense development. One of the major improvements of the QCM technique was provided by Rodahl et. al, who introduced the QCM-D, where the D stands for dissipation monitoring\(^\text{91}\). In addition to mass, the QCM-D also measures the energy dissipation of the adsorbed layer, which can then be used to assess its viscoelastic properties. A special feature of the QCM-D that distinguishes it from other surface sensitive techniques such as surface plasmon resonance (SPR) is that it also detects the water that is associated (oscillating along with) with the surface layer. The amount of water bound to a surface is highly relevant, especially if one wants to get an accurate view of how a specific system behaves in an aqueous environment. The QCM-D technique has thus become increasingly popular, particularly in studies of polymers, and various biochemical processes\(^\text{92-96}\). Ideally, the response from the QCM is directly related to the mass adsorbed onto the sensor surface (and possibly also the
dissipation), but this is rarely the case. Other parameters such as the density and viscosity of the liquid medium surrounding the sensor, surface charge and roughness, pressure waves in the QCM chamber, and the properties of the surface layer, may all affect the response of a QCM sensor. The possibility to use the QCM-D instrument to study zwitterionic sulfobetaine polymers was attempted quite early in my PhD project. Since the QCM-D is capable of detecting both mass and viscoelastic properties, including the amount of water adsorbed by the surface, it appeared to be very suitable for studying the conformation of grafted sulfobetaine polymer chains in various electrolytes. As a second step, we also wanted to investigate their interaction with proteins in order to better understand the unique retention mechanisms of sulfobetaine-type stationary phases. The idea was to prepare a surface layer of sulfobetaine polymers attached to a quartz crystal sensor by surface initiated graft polymerization that could then be studied in different liquid environments. This seemingly straight-forward approach proved, however, to be associated with many practical problems.

Initially, various free radical initiators with thiol functional groups were immobilized onto the gold covered quartz crystal surface to act as “starting points” for surface initiated grafting of SPE monomers. Most of these attempts were unsuccessful however, which we later discovered was due to the instability of the Au-S bond between the crystal and the initiator towards the UV radiation used to initiate the polymerization. We also tried a “grafting to” approach by functionalizing the sensor surface with vinyl group-containing molecules. This actually resulted in polymer formation on parts of the sensor, but the polymerizations were irreproducible and did not yield polymer layers suitable for further QCM-D studies. In a last effort of preparing a sensor surface with zwitterionic functional groups, we prepared a SAM of the detergent Zwittergent 3-14, which resembles the end-group of the SPE monomer. The properties of such a layer, although zwitterionic, are not comparable to those of polymeric sulfobetaines, where the three-dimensional network of inter- and intramolecular attraction and repulsion of charges create a unique ionic environment, which is not exactly mimicked by a monolayer. However, the results from the experiments conducted during that investigation indicated a strange behavior of QCM-D instrument under conductive loading, which appeared to yield biased results in
systems involving charged surfaces and/or electrolytes. A more detailed study of the electrolyte-induced response on the QCM-D, both on neutral and charged surfaces, were undertaken and reported in a manuscript, but as the subject is beyond the scope of this thesis, it will not be discussed further here.

5.2 RAFT-mediated grafting of silica particles with SPE (Paper II)

Stationary phases with zwitterionic functional groups, which contain a positive and a negative charge within a single entity, represent a special case of charged stationary phases. Besides their potential in ion exchange chromatography, surfaces modified with zwitterionic moieties, both of sulfobetaine and phosphocholine type, are biocompatible and resist protein adsorption very well. The ‘soft’ properties of sulfobetaine polymers make them highly interesting in separations of various biomolecules, both in ion exchange mode as well as in HILIC mode for the separation of highly polar analytes. Some recent applications where sulfobetaine-type columns have been successfully used include the separation of opioids and their polar metabolites, separation of glucosinolates from plant extracts, and analysis of amino-hydroxylphenylalanines from melanin pigments.

In Paper II, a new way of grafting silica particles with sulfobetaine polymers was explored. The graft polymerization was performed using RAFT polymerization, in order to attain better control over both the polymerization kinetics, as well as the molecular weight distribution of the polymer chains. RAFT polymerization has previously been used to graft both planar silica surfaces and non-porous silica particles, but its potential use in the preparation of grafted silica particles for chromatographic applications has not gained much attention.

5.2.1 Preparation and characterization of SPE-grafted silica particles

For chromatographic applications, covalent attachment of functional groups to the resin is preferred, as this will avoid problems with
irreproducible functionalization and column bleeding. Grafting of polymers via free radical polymerization is a convenient technique to introduce covalently attached functional groups onto chromatographic supports. In Paper II, a “grafting to” approach was used. The silica particles were first activated with a vinylic silylation reagent to provide anchoring points for the grafting reactions, followed by RAFT mediated graft polymerization of SPE in water. The possibility to use water as a solvent is an advantage, as it reduces the consumption of organic solvents. In order to demonstrate the effect of the RAFT agent on the polymerization kinetics, and thus on the properties of the grafted silica, a batch of particles were grafted without the presence of RAFT agent through a conventional free radical graft polymerization. Measurements of surface area and pore size after grafting shows that the RAFT mediated grafting resulted in higher surface area and larger pore volume compared to the reference conventional grafting. This indicates that the RAFT mediated grafting occurred through a slower and more controlled polymerization procedure, which agrees with the expected suppressing effect of the RAFT agent on the polymerization kinetics. This was also confirmed by elemental analysis. The total carbon content of the RAFT mediated grafted particles was 13.7 %, compared to 16.4 % in the reference particles, which also shows that shorter polymer chains were produced using the RAFT mediated grafting. XPS analysis confirmed that a lower surface concentration of polymer was produced on the particles grafted in the presence of RAFT agent.

5.2.2 Separation of peptides

The chromatographic performance of the grafted silica particles was evaluated in HILIC mode. An initial test of the retention characteristics using a standard test mixture containing toluene, uracil, and cytosine verified that the material was capable of producing separations in HILIC mode. The chromatogram in Figure 10 shows the separation of the test mixture using the RAFT material.
Figure 10. Chromatogram showing the separation of toluene, uracil, and cytosine on a column packed with RAFT-mediated SPE-grafted silica particles using an eluent consisting of 80/20 vol % ACN/25 mM ammonium acetate buffer. Column dimension 50×4.6 mm i.d, eluent flow rate 0.5 mL/min, injection volume 2 μL, UV detection wavelength 254 nm.

The efficiency of the column was good, nearly 100,000 plates per meter was achieved for both uracil and cytosine. The grafted silica particles were also successfully used for the separation of a number of neutral and basic peptides. Basic peptides show particularly poor retention on reversed phase columns due to their polar character. They have, however, a quite strong affinity for bare silica, and the highly hydrophilic nature of the zwitterionic particles thus makes them suitable for separation of basic peptides. Figure 11 shows the separation of bradykinin, Arg-Gly-Glu-Ser, Gly-His-Lys, and Lys-Lys on a column with RAFT grafted silica particles. The peptides eluted according to increasing basicity, which demonstrates that the retention has an ion exchange component. This is a commonly observed feature of zwitterionic separations, but it is also possible that the RAFT mechanism itself is responsible for a more pronounced ion exchange character of the stationary phase. After the RAFT mediated grafting reaction has been completed, the RAFT agent will remain as the distal terminal of the grafted chains and impart a total negative charge of the material because it contains a sulfonate group (Figure 6).
Figure 11. Chromatogram showing the separation of four peptides on a column packed with RAFT-mediated SPE-grafted silica particles using an eluent consisting of 60/40 vol % ACN/100 mM ammonium formate, pH 4. Column dimension 50×4.6 mm i.d., flow rate 0.5 mL/min, injection volume 2 µL, UV detection wavelength 214 nm.

The grafted materials were further evaluated by recording the retention factors (k’) of seven neutral and basic peptides at three eluent strengths. The retention for all peptides increased with the acetonitrile content in the eluent, as expected. In addition to the acid/base character of the peptides, the retention patterns were also strongly affected by their hydrophobicity. Hydrophobic peptides such as Angiotensin II and IV showed a relatively poor retention in all of the eluents, which agrees with their structure. The two grafted materials showed similar retention patterns for all peptides, but neurotensin and prolactin releasing hormone were slightly more retained on the reference column. Bradykinin, which has a pH of 12, had almost no retention on the reference column, but on the RAFT-SPE column a k’ value of 1.25 was achieved in the 60/40 ACN/ammonium formate eluent. This suggests a slightly more anionic character of the RAFT column, in line with the proposed polymerization mechanism discussed earlier. This also demonstrates a “hidden bonus” in graft functionalization of separation materials by controlled polymerization, where proper choice of mediator will not only affect the outcome of the polymerization, but also contribute to the overall selectivity.
5.3 RAFT-mediated grafting of non-porous DVB particles with SPE (Paper III)

Although SPE is readily soluble both in water and in polar organic solvents such as methanol, ethanol, formamide, and isopropanol, grafting of SPE has traditionally been performed in organic solvents, often as a consequence of solvent limitations in the polymerization technique\(^2, 19, 30, 31\). RAFT polymerization is, however, applicable in fully aqueous systems, and solution polymerization of SPE in water has been demonstrated\(^66\). In Paper II, SPE grafts were formed on silica through aqueous RAFT mediated polymerization. The compatibility of SPE polymers with the hydrophilic silica surface makes grafting relatively uncomplicated. A greater challenge is to graft hydrophilic polymers onto extremely hydrophobic materials, such as divinylbenzene (DVB) particles. In Paper III, we successfully accomplished RAFT mediated graft polymerization of SPE on non-porous DVB particles in water. A similar result was achieved with ATRP, and a comparison of the two controlled polymerization techniques was made.

5.3.1 Grafting from dithiobenzoate-modified DVB-particles

The initial attempt at grafting SPE onto DVB particles involved immobilizing a RAFT agent onto the surface prior to grafting. Hydrobrominated DVB particles were reacted with dithiobenzoate to yield particles with R-group supported RAFT agent. The functionalized particles were grafted with SPE in water, to which a small amount of benzyl alcohol had been added to achieve better wetting of the hydrophobic particle surface. The functionalization reaction of the brominated particles to yield dithiobenzoate groups on the surface turned out to be quite straight-forward. XPS analysis of the resulting particles showed the presence of sulfur from the dithiobenzoate on the surface, in addition to significant amounts of oxygen and tertiary nitrogen. The origin of the oxygen signal is still unknown, but the tertiary nitrogen signal was found to be a contamination originating from the hydrobrominated DVB particles. During work-up, the particles were filtrated through a nylon membrane filter which partly dissolved, thus causing a small contamination of amide groups on the particles.
In spite of the unexpected presence of amide group on the original DVB particles detected in the surface analysis, the dithiobenzoate functionalized particles were successfully grafted with SPE in a thermal RAFT mediated graft polymerization. Additional RAFT agent (STPE) was added to the polymerization solution to provide a sufficient concentration of RAFT agent in the water phase. After grafting, the particles were nicely suspended in the water phase, demonstrating the substantial increase in surface hydrophilicity as a result of the incorporation of SPE polymers on the particle surface. The appearance of a quaternary nitrogen signal in the XPS spectrum after grafting also showed the presence of SPE on the surface, as well as sulfur signals from the sulfonate group (1:1 ratio). The tertiary nitrogen signal increased after grafting, which might partly be explained by incorporation of the water-soluble tail fragment of the STPE molecule, or by fragments from the free radical azo initiator.

5.3.2 Direct grafting onto plain DVB particles

The polymerization mechanism in R-group supported RAFT graft polymerization results in transfer of the surface-bound dithiobenzoate into the solution, as radicals add to the C=S bond. This made us question if the modification of the DVB particles with dithiobenzoate was necessary to achieve a RAFT mediated polymerization. Barner et al. have demonstrated successful grafting of styrene directly on crosslinked DVB particles with RAFT agent present only in the polymerization solution. We attempted a similar procedure for the hydrophilic SPE monomer in an aqueous system, and were indeed able to produce SPE polymers on the particles using this approach. XPS analysis showed a 1:1 ratio between quaternary nitrogen and the signal from the sulfonate group, but in contrast to the material grafted from dithiobenzoate functionalized particles, almost no tertiary nitrogen was detected. The first batch of STPE that was synthesized contained a small amount of unreacted dithiobenzoic acid, which appears to have reacted with the particle surface, making them slightly pink in color. When a second batch of STPE without residual dithiobenzoate was used, white particles grafted with SPE were obtained.
5.3.3 Chromatographic application of grafted DVB-particles

Plain DVB particles are very hydrophobic and therefore not suitable for HILIC separations. Since the particles are essentially non-porous, the surface area is quite low and they have a limited capacity. The hydrophilic SPE-grafted particles were evaluated in HILIC mode using a standard test mixture with toluene, uracil, and cytosine, as for the SPE-grafted silica discussed above. Although XPS analysis confirmed that SPE polymers had been grafted onto the particle surface, their chromatographic retention behavior was still dominated by hydrophobic interaction. Apparently, the particles are not covered by a sufficiently dense SPE graft to prevent small molecules from gaining access to patches of the hydrophobic DVB surface. The grafted DVB particles should, however, be potentially useful in separations of large molecules such as proteins, as their size restricts close encounter with the hydrophobic area of the particles.

6. Water structure in sulfobetaine-type stationary phases (Paper IV)

It was quite recently that the biocompatible and non-fouling properties of sulfobetaine polymers were first related to their zwitterionic nature. The same mechanism that results in the biocompatibility of the sulfobetaine polymers is most likely also what causes the unique chromatographic selectivity of stationary phases based on sulfobetaine polymers. A large part of the explanation for the unique properties and behavior of polysulfobetaines is found in how they affect the hydrogen-bonded network of water, and hence its structure, in the vicinity of the polymer chains. The properties of any polymer system in contact with water are largely affected by the interaction of the polymer chains, and a number of techniques have been used to study this, including differential scanning calorimetry (DSC),\textsuperscript{103,104} NMR,\textsuperscript{104-106} and various spectroscopic techniques.\textsuperscript{107-109} Most polymers have been found to more or less disturb the hydrogen bonds between the water molecules.\textsuperscript{104, 106, 107, 110} Kitano et al. have recently specifically investigated the structure of water in aqueous solutions of amphoteric and zwitterionic polymers by Raman
spectroscopy. From the results, they calculated a so-called \( N \) value, which is a measure of the number of hydrogen bonds disrupted due to the presence of one monomer residue. They compared different types of sulfobetaine polymers with other polymers and found that the \( N \) values for electrically neutral polymers were small, whereas for example polyacrylic acid had a high \( N \) value. Sulfobetaine polymers were found to have relatively low \( N \) values, which followed the degree of quaternization (ratio of cationic/anionic groups). This was explained in terms of the hydration of the ionic groups of the sulfobetaine polymers. If the anionic sulfonate group and the cationic quaternary ammonium group are in close proximity, this appears to counteract the electrostatic hydration. The water molecules forming the hydration shells around the ionic groups align themselves so that the partially negative oxygen atom of water is attracted by the ammonium group, and the partially positive hydrogen atoms by the sulfonate group. Although the water structure is apparently disturbed, the effect was smaller than for other types of polymers. The difference between sulfobetaine polymers and the zwitterionic polymer poly(2-methacroyloyloxyethyl phosphorylcholine), polyMPC, which has been shown not to disturb the hydrogen network of water at all, was ascribed to the inhibition of tight ionic associations in polyMPC by the bulky phosphate group in the MPC residues. The same authors later went on to study the structure of water in several types of thin sulfobetaine polymer films using ATR-FTIR, and found that the polymers had a small effect on the structure of water in the films, analogous to the results for aqueous polymer solutions.

Water present in polymer systems may be divided into three types, free water resembling ordinary bulk water, freezable bound water, which has a slightly shifted phase transition temperature relative to bulk water, and non-freezing water, which is defined as bound water that does not freeze in the normal temperature range expected for bulk water. NMR is a very useful technique for studies of water structure, not only in order to detect and quantify the relative amounts of free and bound water in a polymer system, but also to study more complex events such as mobility of water molecules within a material and possible intermolecular exchange.
In Paper IV, high field $^2$H NMR was used to study the phase transitions of water in porous silica particles at temperatures below 0 °C, both in unmodified porous silica with different pore sizes and in particles grafted with sulfobetaine polymers. By first cooling samples that had been equilibrated with a certain amount of $^2$H$_2$O to temperatures approaching -80 °C, and then thawing them, the amount of unfrozen water in the samples at different temperatures could be recorded.

For the unmodified silica samples, there is a thin water layer close to the surface that starts to thaw at temperatures as low as -65 °C to -50 °C, although most of the water in the pores melts at about 10 to 20 °C below the melting point of bulk water (Figure 12, upper part). The effect of pore size on the amount of unfrozen water in the samples is evident. The largest amount of unfrozen water is found in the 60 Å silica and then decreases as the pore size increases, as expected from the relative fraction of water that is in close proximity to the silica surface in each sample. Compared to the unmodified silica samples, the water in the sulfobetaine grafted silica was much more affected, which was reflected as a clearly higher amount of unfrozen water throughout the whole temperature range (Figure 12, lower part). For example, the zwitterionic silica prepared by RAFT polymerization contained approximately 17 % unfrozen water at -50 °C, compared to only 5 % in the unmodified 100 Å silica. Another difference between the grafted and unmodified silica samples is the shape of the thawing curves. While still in the low temperature region, only small amounts of water melted in the unmodified silica, and the major part of the water melted at temperatures above -13 °C. The melting of the water in the sulfobetaine grafted silicas occurred in a more continuous manner in the lower temperature region, and the slope of the thawing curve in the higher temperature interval was less steep.
Figure 12. Plots of the fraction of unfrozen water (normalized against the amount detected at -1 °C) observed during the thawing cycle for unmodified (upper) and zwitterionic grafted silicas (lower).

A comparison of the silicas prepared by RAFT polymerization (SPE-RAFT) and uncontrolled free radical polymerization (SPE-Ref), both based on 100 Å silica, shows that the material prepared by uncontrolled polymerization contains the highest amount of unfrozen water of all the grafted materials. The RAFT material is expected to have a more even coating of polymer chains, and should thus have a
steeper thawing curve in the region above -20 °C, which is indeed what was observed.

Another factor affecting the amount of unfrozen water in the zwitterionic materials is the length of the grafted chains. The SPE-Ref material, prepared by an uncontrolled polymerization, contains more carbon than the material obtained via the RAFT procedure, and thus has a higher polymer content. This is reflected in the plots showing the amount of unfrozen water, where the SPE-Ref material contains the largest fraction of unfrozen water of all the grafted materials.

In order to correlate the retention characteristics of the zwitterionic silica with the properties of water in the materials, columns packed with the grafted silica and the 100 Å unmodified silica was evaluated in HILIC mode. A number of polar analytes were injected on the columns and the retention factors (k’) after separation in HILIC mode were recorded. For chromatographic applications, the properties of the stationary phase are of course what determine the retention pattern of an analyte. Data from the thawing curves were used to estimate the amount of non-freezing and bound freezeable water in each grafted phase. Non-freezing water may be considered to be part of the stationary phase and thus contribute to the overall retention in a column. The difference in the amount of non-freezing water, and thus water structure, between the grafted phases provided agreed well with the retention characteristics of the tested analytes. An increasing amount of unfrozen water led to stronger retention. For materials of different pore size, the properties of the water enriched stationary phase were less crucial, and comparisons should thus primarily be made between materials of similar pore size.

In the future, extended NMR studies on the state of water in these materials would be of interest, particularly aiming at revealing the diffusional movement of water molecules in the stationary phase.
7. Concluding remarks

In the thesis, RAFT polymerization has been used to synthesize sulfobetaine polymers in different ways. Paper I describes the UV-initiated polymerization of SPE in aqueous solution to produce short telomer chains. The polymerization kinetics was clearly affected by the presence of RAFT agent in the solution. The polymerization was fast and efficient, yielding a monomer conversion of over 90% in about 90 minutes. Although a high concentration of RAFT agent was used, the pronounced rate retardation that is commonly observed in dithiobenzoate-mediated RAFT polymerizations was however absent.

Paper II deals with graft polymerization of SPE onto silica particles, also using RAFT polymerization in aqueous solution. The presence of RAFT agent resulted in a slower and more controlled polymerization compared to a reference batch without RAFT agent. The material was successfully used to separate peptides in HILIC mode.

Highly hydrophobic DVB particles were grafted with SPE in Paper III. Two alternative strategies were used. In the first attempt, RAFT agent was immobilized onto the DVB particles surface, followed by grafting in water solution. Grafting was also performed directly on DVB particles. Both routes resulted in the production of SPE grafts on the particles. The latter approach resulted in varying amounts of polymer grafted to the surface as function of the ratio of monomer to RAFT agent, indicating the possibility to control the molecular weight of the grafts.

The water structure near silica-based sulfobetaine type stationary phases was investigated in Paper IV using $^2$H NMR. The zwitterionic sulfobetaine polymers clearly disturb the water at the stationary phase interphase. This was observed as a significantly higher portion of unfrozen water even at temperatures approaching -80 °C in the grafted materials as compared to bare silica particles of similar pore size, which also affected the chromatographic behavior of the sulfobetaine modified stationary phases.
8. References

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"When you look at yourself from a universal standpoint, something inside always reminds or informs you that there are bigger and better things to worry about."

Albert Einstein