Purification and surface modification of polymeric nanoparticles for medical applications

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Performed at
SINTEF
Materials and Chemistry
Trondheim, Norway

2008-03-03
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SINTEF Materials and chemistry
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Polymeric nanoparticles are potential candidates as carriers for pharmaceutical agents. Development of such nanoparticles generally requires molecules immobilized on the particle surfaces to ensure biocompatibility and/or targeting abilities. Following particle preparation and surface modification, excess reagents must be removed. Ultracentrifugation, which is the most widely used purification technique as per today, is not feasible in industrial applications. In this diploma work, tangential flow filtration is studied as an alternative purification method which is better suited for implementation in a large-scale process.

Comparison of ultracentrifugation and tangential flow filtration in diafiltration mode for purification of nanoparticles, indicate that they are comparable with respect to particle stability and the removal of the surfactant SDS from methacrylic anhydride nanoparticles. The purification efficiency of tangential flow filtration is superior to that of ultracentrifugation. Conductivity measurements of filtrates and supernatant liquids show that a stable conductivity value can be reached 6 times faster in filtration than in centrifugation with equipment and settings used. This conductivity arises from several types of molecules, and the contribution from surfactant molecules alone is not known. However, protein adsorption on the particles indicates successful removal of surfactant.
Abstract

Polymeric nanoparticles are potential candidates as carriers for pharmaceutical agents. Development of such nanoparticles generally requires molecules immobilized on the particle surfaces to ensure biocompatibility and/or targeting abilities. Following particle preparation and surface modification, excess reagents must be removed. Ultracentrifugation, which is the most widely used purification technique as per today, is not feasible in industrial applications. In this diploma work, tangential flow filtration is studied as an alternative purification method which is better suited for implementation in a large-scale process.

Comparison of ultracentrifugation and tangential flow filtration in diafiltration mode for purification of nanoparticles, indicate that they are comparable with respect to particle stability and the removal of the surfactant SDS from methacrylic anhydride nanoparticles. The purification efficiency of tangential flow filtration is superior to that of ultracentrifugation. Conductivity measurements of filtrates and supernatant liquids show that a stable conductivity value can be reached 6 times faster in filtration than in centrifugation with equipment and settings used. This conductivity arises from several types of molecules, and the contribution from surfactant molecules alone is not known. However, protein adsorption on the particles indicates successful removal of surfactant. Conductivity and tensiometry were evaluated as potential methods to quantify surfactant in solutions, but both proved unsatisfactory.

Using bovine serum albumin as a model protein, the extent of immobilization to nanoparticles is evaluated at different pH. A maximum amount of 6,8 mg/m² is immobilized, whereof an unknown part is covalently bound. This coverage is achieved at pH 4,0 and is probably partly due to low electrostatic repulsion between particle and protein. An estimation of 2,0 µmol covalently bound BSA per gram of nanoparticles corresponds to 5,3 mg/m² and a surface coverage of 76%. Removal of excess reagents after surface modification is done with ultracentrifugation instead of filtration, as particle aggregates present after the immobilization reaction might foul the membrane.
Acknowledgements

I would like to thank Per, Ruth, Heidi and all other at SINTEF Materials and Chemistry for advice and assistance during the course of this project. Kåre Larsson and Christer Tungard at Millipore Ltd. are acknowledged for guidance with ultrafiltration equipment and procedures. I also thank Inger Lise Alsvik at the Institute of Chemistry, NTNU, for interesting discussions and help with conductometry measurements, as well as Erland Nordgård at Uglestad Laboratory for demonstrating the tensiometer.

Kajsa Uvdal and Bo Liedberg, thank you for introducing me to surface science. Mum, dad and granddad – this thesis concludes 4,5 years of studies that in several aspects might not have been if it weren’t for you. Thank you for everything. Børge, thank you. I would not have been able to work as continual with this project if it weren’t for your ability to get me out of the apartment, all days of the week.
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<tr>
<td>BSA</td>
<td>Bovine serum Albumin</td>
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<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
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<tr>
<td>DV</td>
<td>Diavolume</td>
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<tr>
<td>EDC</td>
<td>1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride</td>
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<tr>
<td>EGDMA</td>
<td>Ethylene glycol dimethacrylate</td>
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<tr>
<td>FTIR</td>
<td>Fourier transform IR</td>
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<tr>
<td>IR</td>
<td>Infrared</td>
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<tr>
<td>MAAH</td>
<td>Methacrylic anhydride acid</td>
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<tr>
<td>MPS</td>
<td>Mononuclear phagocytic system</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
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<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
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<tr>
<td>NP</td>
<td>Nanoparticle</td>
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<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
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<tr>
<td>PVA</td>
<td>Poly(vinyl alcohol)</td>
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<tr>
<td>PVV</td>
<td>Poly(vinyl pyrrolidone)</td>
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<tr>
<td>RPM</td>
<td>Rotations per minute</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SN</td>
<td>Supernatant liquid</td>
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<tr>
<td>TFF</td>
<td>Tangential flow filtration</td>
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<tr>
<td>TMP</td>
<td>Transmembrane pressure</td>
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1 Introduction

1.1 Background
A research area that has drawn a lot of interest over the past few decades is nanoparticles as drug delivery devices. Pharmaceutical agents encapsulated in or adsorbed to nanosized particles enable targeted administration, minimization of side effects, and protection of the drug during delivery. By releasing the drug solely at the site of disease, lower doses can be administered as only a minor part will be lost to other parts of the body. Toxic drugs such as cytostatics will affect only the targeted tissue, and thereby side effects are minimized.

There are many obstacles to overcome before targeted drug delivery can be realized. The particles must be safely eliminated from the body, for instance by degradation or filtration through the renal system. Other required nanoparticle characteristics are biocompatibility, the ability to release pharmaceutics in a controlled fashion at the wanted location, and to evade recognition from the immune system. Some of these characteristics can be obtained by modifying the particle surface. Surface bound polymers may give protection from the immune system, and the ability to reach a predefined target may be achieved by immobilizing specific target proteins on the nanoparticle surface.

1.2 Purpose
Preparation and surface modification of nanoparticles leave excess reagents in the nanoparticle solution. These compounds have to be washed away to enable the particles to be further surface modified and also to be used in medical applications. The main purpose of this thesis has been to find and evaluate a purification method for nanoparticles that is well adapted to industrial processes. Good purification is defined by a high yield, physical stability of product and a low level of impurities.

Immobilization of antibodies on the particle surface is one way to achieve targeting properties. In this study, a model protein was used to evaluate the extent of protein attachment on the nanoparticle surface after purification and chemical coupling at different conditions.
The particles used are model particles not intended for medical use, and are only used for studying purification and surface modification. Other areas of interests regarding nanomedicine, such as biodegradability, toxicity and the encapsulation of drugs, are not comprised in this project.

1.3 Sources of information

Procedures for nanoparticle preparation and surface modification followed protocols previously used at SINTEF. Scientific articles were used to study the theory. Ideas on modification of the protein immobilization method were found on the webpage Pierce Biotechnology ([27]). Scientific articles have been used in background studies on nanomedicine and when deciding on which purification method to study in this project. For the method of choice, description of experimental setup varied a lot between the different articles. Therefore, the articles were only used for general information and for comparison with obtained results. All specific information on apparatus and guidance with settings was obtained from meetings and telephone conversations with Millipore employees, as well as technical briefs and data sheets. Theory on analytical methods was found mainly on instrument dealers’ webpages, such as Malvern Instruments, Cole Parmer and KSV Instruments.

All work has been done at SINTEF Materials and Chemistry in Trondheim, Norway, under supervision from Per Stenstad, Ruth B. Schmid and Heidi Johnsen.
2 Theory

2.1 Nanoparticles as drug delivery devices

Particles in the nanometric range ($10^{-9}$ m), namely nanoparticles, have a multitude of potential uses in everything ranging from computer components to new super strong materials. An interesting application of solid or hollow nanoparticles is targeted delivery of medicine. Nanoparticles prepared from polymers, lipid molecules or a multitude of other materials are modeled to carry medicine through the blood stream or gastrointestinal tract, and release its content at the site of disease.

Nanoparticle encapsulation is a method to protect therapeutic agents during delivery, but also to protect the body from highly toxic drugs, such as cytostatic agents. By encapsulating the drugs in or adsorbing them onto the particles, controlled release is possible. This can be achieved either by diffusion out of the particle over a period of time, or release at a specific site as the particle is degraded. The use of nanoparticles instead of microparticles gives a larger surface/volume ratio. This increases the diffusion efficiency of drugs out of the particles, as well as the interaction with cells and tissue. Their sub-cellular size enables them to penetrate deep into tissue, and enhances cellular uptake [4].

Research on drug carrying particles began with vesicles of phospholipids, also known as liposomes. Since phospholipids are present in cell membranes, their biological compatibility is good [2]. A phospholipid molecule has a polar head and a hydrophobic tail. By forming micelles, they create a hydrophobic environment within the particles, and present a hydrophilic surface to the surroundings. Hydrophobic drugs can be solved among the molecule tails and thereby transported in the blood stream. Although liposomes possess many important features, such as relatively simple surface modification and invisibility to the immune system, they tend to have insufficient loading capacities and suffer from uncontrolled release of water soluble drugs when in the circulatory system, as well as poor storage stability. Nevertheless, there are several liposome-based agents on the market today.[1]
Nowadays, other materials are studied as possible drug carrier candidates, such as chitosan molecules, fullerenes and polymeric nanoparticles [1],[2]. By synthesizing polymeric nanoparticles instead of using liposomes, more stable systems with high loading capacities can be obtained. Both biodegradable and non-degradable polymer nanoparticles are potential candidates as delivery devices [3].

2.1.1 Surface modification
An important feature for nanoparticles to be introduced in the blood stream is “stealth” – invisibility to the body’s natural defense system. Unless the particles are modeled to escape recognition, the mononuclear phagocytic system, MPS, eliminates them from the blood stream efficiently [5]. Longer circulation times increase the probability for the nanoparticles to reach their target. Small particles (<100 nm) with a hydrophilic surface have the greatest ability to evade the MPS [3]. To further increase circulation times, the particles can be coated with molecules that provide them with a hydrophilic protective layer, such as poly(ethylene glycol), PEG, or poly(vinyl pyrrolidone), PVV. This steric layer prevents macromolecules to interact with the particle, even at low surface coverage.[7]

For particles to release their load at a specific site, they first have to accumulate there instead of flowing round after round in the circulatory system. This can be ensured by passive or active targeting. An example of passive targeting is when nanoparticles accumulate at tumor sites because of the leaky vasculature that often characterizes tumor tissue. In active targeting, the nanoparticles carry targeting molecules on the surface that are able to interact with the surrounding tissue [8]. Cancer cells express specific antigens and also have surface folate receptors accessible from the circulatory system. As no healthy cells have these characteristics, active targeting can be achieved by immobilizing antibodies onto the nanoparticle surface that interact with these macromolecules.[3]

2.2 Nanoparticle preparation: Miniemulsions and hydrolysis
There are numerous different methods to produce nanoparticles. Polymeric nanoparticles can be synthesized either by polymerization of monomers, or by manipulating existing polymers [9]. The miniemulsion method polymerizes monomer droplets in a solution, creating particles in the nanometer range.
An emulsion consists of small droplets of one phase in another phase, either a dispersed organic phase in a continuous water phase or vice versa. A hydrophobic solvent will form oil droplets in a water based buffer. Addition of a hydrophobic monomer to such a system will result in oil droplets containing monomer, dispersed in a continuous aqueous phase. The low solubility of monomer in the hydrophilic phase prevents migration and ensures little or no monomer outside the droplets. A stabilizer is added to prevent coalescence of the droplets. The miniemulsion method uses high shear, e.g. by ultrasound, to create very small droplets. Addition of initiator to the solution will start polymerization of the monomers. Each monomer containing droplet will then serve as a mini reactor during polymerization, resulting in one nanoparticle per droplet. [12],[16]

Surface charges prevent the particles to aggregate, by means of electrostatic repulsion. They can also serve as anchors when chemically modifying the surface. Negatively charged surfaces can be achieved either by using monomers that give hydroxyl groups after polymerization, or by introducing hydroxyl groups by hydrolysis of the particles (see Figure 1).

![Figure 1: Hydrolysis of an anhydride group. In MAAH nanoparticles, it breaks up the MAAH polymers and creates negatively charged carboxyl groups.](image-url)
Hydrolysis of monomers such as MAAH renders them water soluble. To prevent the particles to dissolve after hydrolysis, polymerization is done in the presence of a cross linking molecule that is not affected by hydrolysis (see Figure 2).

![Polymerization and Hydrolysis Diagram]

- MAAH monomer
- EGDMA monomer

**Figure 2**: Schematic picture of polymerization and hydrolysis. EGDMA polymers hold the structure together.

### 2.3 Purification

The purification of nanoparticles intended for medical use is of great relevance. Depending on the method of preparation, different substances will be present in the nanoparticle solution and adsorbed to the particles. The miniemulsion method leaves surfactant molecules adsorbed to the surface (see Figure 3). Removal of these molecules is of great relevance, as they obstruct the chemical reactions necessary for surface modification of the particles, in addition to being possibly toxic. A dilemma here is that the particles might not be stable without the surfactant present on the surface, which may result in particle aggregation.

Other potentially toxic compounds such as rest monomers, particle aggregates and initiators must also be removed prior to administration, to ensure biological tolerance. Furthermore, the physical behaviour of the nanoparticles might be altered by the presence of these compounds.[9]
Figure 3: Schematic picture of hydrolyzed particle, with COO\(^{-}\) groups and surface adsorbed surfactant.

Purification is necessary not only after preparation, but also after surface modification, to remove excess reagents. Each modification step is followed by extensive washing. An efficient purification method that removes a satisfactory amount of unwanted substance, without affecting the particles in a negative way, is important for successful preparation of surface modified nanoparticles.

Dialysis, gel filtration and ultracentrifugation are commonly used purification methods, but they all have the disadvantage of a problematic scale up process. Ultrafiltration, on the other hand, is a purification method that can be totally automated, is highly cost effective for large batches and therefore is possible to implement in industrial processes.[9],[10]

Heydenreich et. al. (2003) compared ultrafiltration in a stirred cell, ultracentrifugation and dialysis to remove the surfactant polysorbate 80 from lipid nanospheres. They found that the filtrated particles aggregated after a 1-week storage, something Miglietta et. al. (2000) did not report as a problem. Ultrafiltration in a stirred cell was also found unsatisfactory for removal of the surfactant in question.

Dalwadi et. al. (2005) compared filtration in a centrifugation device with ultrafiltration (TFF, see 2.3.2), dialysis and ultracentrifugation. In contrast to
Heydenreich et. al., they recommend ultrafiltration, as it is more efficient than dialysis and the filtration-centrifugation-setup, and gentler to the particles than ultracentrifugation. Another group reporting positive results from ultrafiltration is Limayem et. al (2004). Both these groups remove PVA (polyvinyl alcohol) from polymeric nanoparticles, using tangential flow filtration in diafiltration and concentration mode (see 2.3.2).

2.3.1 Ultracentrifugation
The most common way to remove large quantities of process impurities is by ultracentrifugation and subsequent redispergation in clean buffer or water. This method is simple, but it sometimes results in aggregation of nanoparticles due to centrifugation forces, with difficulties in redispergation as a consequence. It might also cause loss of finer nanoparticles in the supernatant liquid, resulting in a low yield [9]. Ultracentrifugation can remove excess reagents from small batches of nanoparticles, but is not a suitable purification method for industry applications [10].

2.3.2 Ultrafiltration
Tangential flow filtration (TFF, also known as crossflow filtration) is a form of ultrafiltration that is usually used to concentrate, separate and clarify proteins [17]. As nanoparticles are in the same size range as proteins, tangential flow filtration has previously been studied as a possible separation and purification method [9],[10],[18], [19].

The difference between normal (dead-end) flow filtration and tangential flow filtration is the direction of the flow (see Figure 4). By pumping the feed tangentially along the membrane, the build up of particles on the membrane that can pose a problem in normal flow filtration, is minimized. Particles that are too large to pass the membrane are swept along, instead of accumulating at the membrane surface. This minimizes concentration polarization and membrane fouling, which can decrease washing efficacy and filtrate flux, and are common problems in normal flow ultrafiltration. [9],[17]
Figure 4: Comparison of normal (dead-end) flow filtration and tangential flow filtration. With courtesy of Millipore Corporation, U.S.A. [17]

During filtration, the starting solution (feed) will be divided into two solutions, the retentate and the filtrate. The retentate (also known as concentrate) is a solution with all particles that are too large to pass through the membrane and therefore are retained. In normal flow filtration, the retentate consists only of large particles, but because of the tangential flow, some solute and small molecules will be pushed past the membrane, and make the retentate a solution. The retentate can be either collected in a separate vessel, or returned to the feed vessel. The solution passing through the membrane, the filtrate, is also known as permeate, as the membrane is permeable for it (see Figure 6).

Tangential flow filtration can be performed in either concentration or diafiltration mode (see Figure 5). In concentration mode, the feed volume is reduced by filtration, and thereby the particle concentration increased. During diafiltration, on the other hand, the solution volume is kept constant by adding new buffer as filtrate is removed. By doing so, buffer exchange is possible. As the buffer is exchanged, all undesired species that is dissolved in it, will be removed. This is why filtration is a potentially good method for purification of nanoparticles.
Figure 5: Comparison of concentration (A) filtration and continuous diafiltration (B). NP represents the nanoparticles and SDS the solute to be removed.

Diafiltration can be done in either discontinuous or continuous mode. In continuous mode, new buffer is added at the same rate as filtrate is being produced, whereas it is added after defined intervals in discontinuous mode. In diafiltration, diavolumes is a measure of buffer exchange extent. One diavolume (1 DV) has been processed when a buffer volume equal to the initial feed volume has been added.

Theoretically, there will be a linear decrease in solute content in the concentration mode, and an exponential decrease in diafiltration mode. This model is applicable for all solutes to be eliminated, but with different slopes depending on the permeability of the solute. 100% permeability means that all solute passes through the membrane, which is the case for salts, solvents and buffers. This means that more than 99.5% of a totally permeable solute will be removed after 6 DV in continuous diafiltration mode. For molecules with a permeability of 75%, 8 DV must be run before the same amount of solute is removed from the solution. [25]

Four important parameters in tangential flow filtration is the feed flow, the transmembrane pressure, the filtrate flux and the solution concentration. A high feed flow will reduce the concentration gradient at the membrane, giving lower risk of fouling, but also increase the retention of most components. As the feed flows along
the membrane, the applied pressure will force a portion of the solution through the membrane over to the filtrate side. This pressure is called the transmembrane pressure, TMP, and it affects the amount of solute removed from the solution, but also the fouling of the membrane. The TMP can be controlled by means of a valve at the retentate end of the filter (see Figure 6). There is a pressure drop from the feed end of the membrane to the retentate end. TMP is therefore calculated as the average pressure over the membrane; \( \frac{P_f - P_r}{2} \), where \( P_f \) is the pressure at the feed inlet, and \( P_r \) the pressure at the retentate outlet. Filtration with a concentrated solution reduces the required buffer volume and saves time, but only to a certain point. Too high concentrations will make the flux rate too slow because of high viscosity and fouling, and actually slow down the process. The filtrate flux is a measure of the amount of filtrate generated per membrane area. All these parameters influence each other as well as the result. [17],[25]

![Figure 6: Schematic picture of a TFF system in diafiltration mode. With courtesy of Millipore Corporation, U.S.A. [17]](image)

The membrane pore size is given in molecular weight cut off (MWCO) and indicates the weight of the retained proteins. A pore size in nanometers can be approximated using electron microscopy. Using the filtration equipment with two membranes of different pore sizes enable separation of nanoparticles of a certain size range from the suspension. Firstly, a membrane with large pores is used to remove aggregates and big nanoparticles, leaving small, single nanoparticles in the filtrate. By filtering this
filtrate with a membrane of smaller pore size, molecular impurities will be washed away, and the final product will be in the retentate. [9], [19]

2.4 Protein immobilization

Proteins such as immunoglobulins can be coupled to nanoparticles to achieve targeting abilities. One way of immobilizing proteins is by coupling primary amines in the proteins (only in the amino acid lysine and the protein N-terminal) to carboxyl groups present on the particle surface.

This is done using EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride). EDC activates the carboxyl groups by forming an unstable reactive ester, which in turn can react with primary amines to a stable amide bond. In the presence of sulfo-NHS (N-hydroxysulfosuccinimide), an intermediate that is much more stable is formed, and a higher degree of protein immobilization is obtained (see Figure 7). [27]

![Figure 7: Possible reaction paths. EDC activates a surface carboxyl group, which can form a stable amide bond with a primary amine, without (1) and in (3) the presence of sulfo-NHS, or be regenerated (2). Redrawn from figure 2 in [27].](image-url)
2.5 SDS – sodium dodecyl sulfate

The surface active agent used to stabilize the emulsion during polymerization in the miniemulsion method will be present in the final solution, both adsorbed to the particles, and free in the solution. The surfactant sodium dodecyl sulfate (SDS) has been used to stabilize all emulsions in this project. By covering the particles, it renders protein coupling more difficult due to steric hindrance. In addition to this problem, SDS is a toxic substance. For medical applications, it must be removed down to non-toxic levels prior to administration, or substituted with another surfactant that is approved for medical use.

SDS is an anionic detergent with a polar head group and a nonpolar hydrocarbon tail (see Figure 8). In a water/oil or water/air interface, it will orient itself with the hydrophilic part in the water and the hydrophobic part in the oil or air, thereby influencing the surface tension. SDS will also affect the conductance of a liquid. The negatively charged sulfate group and its positive sodium counter ion will both contribute to a higher conductance for a SDS solution than for pure water.

![Sodium dodecyl sulfate with hydrophilic head group to the right and hydrophobic tail to the left.](image)

**Figure 8:** Sodium dodecyl sulfate with hydrophilic head group to the right and hydrophobic tail to the left.

The influence SDS concentration has on conductance and surface tension of a liquid can be seen in Figure 9. At the critical micelle concentration, CMC (vertical line), there is often an abrupt change of the physical properties. The CMC is the concentration when the molecules do not float around separately in the solution anymore, but arrange themselves in micelles. This concentration is temperature dependent and is lowest at 29° C according to Garcia et. al. (2000). They also say there is a 20% increase of the CMC between 20° and 60° C.
As seen in Figure 9, the sensitivity of both conductometry and tensiometry measurements will be highest at concentrations under the CMC, due to the higher slope in this range.

![Graph showing conductance and surface tension as a function of SDS concentration. CMC indicated by the vertical line. Redrawn from figure in [22].](image)

**Figure 9**: Conductance and surface tension as a function of SDS concentration. CMC indicated by the vertical line. Redrawn from figure in [22].

### 2.6 Analytical methods

Characterization of nanoparticles can be done with respect to their zeta potential, size and size distribution, as well as their composition and surface bound molecules.

The surface active properties of SDS and its charged head group possibly enable quantification of SDS in solutions by tensiometry and conductometry measurements.

#### 2.6.1 Zeta potential

Most particles in a colloidal system have a charged surface due to protonatable or ionizable surface groups. These charged surfaces, whether positive or negative, give rise to an electric double layer as counter ions concentrate around the particles. The counter ions closest to the surface will follow the particle motions. This layer of closely associated ions is called the Stern layer and is delimited by the surface of hydrodynamic shear, also called the slipping plane. The potential at this plane is defined as the zeta potential (see Figure 10).
Zeta potential is a physical property of particles in suspensions. It depends on charged surface groups and gives an indication of the stability of a suspension. Systems with a low zeta potential value, whether positive or negative, have low repulsion between the particles, which will eventually aggregate. For a system to be considered stable, a zeta potential of around (±) 30 mV is necessary. Zeta potential is highly pH-dependent and measurements should be made in buffered solutions. [28]

2.6.2 Photon Correlation Spectroscopy

Photon correlation spectroscopy (PCS) is also known as Dynamic Light Scattering (DLS). It is a method that utilizes the Brownian motion of particles and molecules in suspension, to measure their size and size distribution. Proteins, micelles, nanoparticles, emulsions and polymers can all be analyzed with photon correlation spectroscopy.

All particles and molecules that are dispersed or dissolved in a liquid will be hit by solvent molecules that move due to thermal energy. This collision induced motion is called Brownian motion. When illuminating the solution with laser light, the light scatter intensity will fluctuate in respect to how fast the particles move. Since small particles will move faster after collisions than big ones, light scatter analysis give information on particle size and size distribution.

Figure 10: Schematic representation of zeta potential. With courtesy of Malvern Instruments Ltd. UK [28].

- 15 -
The particle velocity depends not only on particle size, but also on surface structure and the concentration of ions in the medium. The reported size is therefore not the core size, but the hydrodynamic diameter (see Figure 11). Molecules attached to the particle surface will increase the hydrodynamic diameter. This theoretically gives the possibility to follow surface modification of particles. [29]

![Figure 11: Schematic picture of hydrodynamic diameter of particle. With courtesy of Malvern Instruments Ltd. UK [29].](image)

Photon correlation spectroscopy measurements give an intensity distribution. This distribution can, by the use of mathematical models and a correlation function, be converted to a volume distribution, which can be further converted to a number distribution. However, number distributions are of limited use, as small errors in gathering data for the correlation function, will lead to huge errors in distribution by number. [30]

### 2.6.3 FTIR

Fourier Transform Infrared spectroscopy (FTIR) can be used to study the molecular composition of a compound. Infrared electromagnetic radiation is the range between the visible and microwave regions, but of greatest practical use is the limited spectrum part between 4000 cm\(^{-1}\) and 666 cm\(^{-1}\). In this wavelength region, molecules absorb and convert the energy to molecular vibrations. Each vibration change is accompanied with a number of rotational energy changes, and these vibration-rotation changes give a spectrum with broad bands instead of lines.
The frequency of absorption depends on the force constants of the bonds within the molecule, the relative masses of the atoms involved and the molecule geometry. There are two types of molecular vibrations; stretching and bending modes. Only vibrations resulting in a change of dipole moment will be observed in the infrared. Functional groups with a strong dipole therefore give rise to strong absorptions. [23]

Extensive tables that show the absorption frequencies of chemical bonds are used as guides when interpreting infrared spectra.

For more detailed theory on infrared spectroscopy, refer to Silverstein et. al. (1980).

### 2.6.4 Ninhydrin staining

Ninhydrin staining can be used to quantify the amount of protein in a solution, or immobilized on a particle surface. Ninhydrin solution reacts with primary amines in proteins (only in the amino acid lysine) attached to the particle surface, creating a blue product that can be detected by UV/vis spectroscopy at 570 nm (see Figure 12) [31]. The absorption is proportional to the amount of primary amines in the solution, enabling quantification of protein by using a standard curve.

Before measuring the absorption, the particles in the solution have to be spun down as not to disturb the measurement.
2.6.5 Surface tension

Surface tension is a force that strives to minimize the area of a liquid interface. It arises due to molecular interactions in the liquid, and anything that affects these interactions will also influence the surface tension [32]. Surface active agents (surfactants) have a tendency to accumulate at interfaces due to their amphiphilic nature, resulting in a surface tension decrease.

Surface tension can be measured as energy per area or force per length, using either a Wilhelmy plate or a Du Nouy ring. The units are equivalent and surface tension is usually expressed in mN/m. Using the Du Nouy ring, the maximum force exerted by the liquid on the ring, just before disruption of the liquid meniscus underneath it, is measured (see Figure 13). [33]
2.6.6 Conductance

Conductivity is a solution’s ability to conduct electrical current, measured in Siemens (S), but usually expressed in S/cm. The conductance of a solution is proportional to its ion concentration, and can therefore be used as a measure of the amount of charged molecules in a solution. The ion mobility, i.e. the size and charge of the dissolved ions, are also significant for the conductance of a solution.[34]

Conductance measurements can be done with a conductometry cell. Conductometry is a quantitative analytical method that gives no information on what kind of ions are present, only the amount. Conductivity measurements are temperature dependant, and corrections must always be done. A conductometry cell usually has two electrodes and a temperature sensor and can be either a probe or a flow-through cell.[35]
3 Materials

3.1 Chemicals
All chemicals used are listed in appendix A.
All buffers and other solutions used are listed in appendix B.

4 Methods
To prepare purified protein-coupled nanoparticles, the procedure is as follows:

- Nanoparticle synthesis
- Hydrolysis of anhydride groups
- Purification – removal of surfactant
- Activation of surface carboxyl groups
- Protein coupling
- Purification – removal of excess reagents

4.1 Synthesis of nanoparticles
The miniemulsion method (see 2.2) was used to synthesize particles with a diameter of 100-200 nm. Nanoparticles of this size are relatively easy to work with, and will not aggregate as easily as smaller nanoparticles. Four separate batches (B1-B4) were prepared during the course of this project.

Equal amounts of methacrylic anhydride acid (MAAH) monomer and cross linker ethylene glycol dimethacrylate (EGDMA) were dissolved in hexadecane, constituting the hydrophobic phase. The hydrophilic phase was prepared of 0,1 M acetate buffer at pH 4,0 with SDS (3,05 g/l) as surfactant and KI (0,30 g/l) as initiator. The final emulsion consisted of 20% hydrophobic phase.

The two phases were mixed on magnetic stirrer and emulsified using an ultra turrax for 2 min at 200 W. The polymerization vessel was exposed to nitrogen gas for 5 minutes to remove all oxygen, which otherwise would disturb the reaction. The emulsion was bubbled with nitrogen gas for 5 minutes and poured into the
polymerization vessel. Polymerization was done at 60°C for at least 6 hours while stirring at 150 rpm.

When large aggregates (~5000 nm) were present after polymerization, the nanoparticle solution was filtered through glass wool in a funnel.

4.2 Characterization of nanoparticles

Approximately 1 ml of polymerized solution was dried at 80°C overnight to gravimetrically determine the nanoparticle content. This was done also after purification to assess nanoparticle loss.

The solution was studied before and after polymerization with a Zeiss light microscope with 1,6 x 63 enlargement. The droplet and particle size was measured in water before and after polymerization, respectively, using a Malvern Zetasizer Nano ZS. The zeta potential after polymerization was measured with the same instrument to determine the particle stability. To ensure a stable pH, zeta potential measurements were done in 0,01 M phosphate buffer at pH 7,2.

A Perkin Elmer Spectrum One FTIR spectroscope was used to study the chemical composition of the particles. The particles were studied both as a water based paste (precipitate after centrifugation, at 2800 - 700 cm\(^{-1}\)) and dried powder (at 4000 - 650 cm\(^{-1}\)). Water absorbs above 3000 cm\(^{-1}\) and therefore only dry samples were measured above 2800 cm\(^{-1}\).

4.3 Hydrolysis

Hydrolysis of the particles cleaves MAAH polymers, at the same time creating negatively charged carboxyl groups (see 2.2). These carboxyl groups stabilize the particles by creating a zeta potential, work as anchors at immobilization reactions, and possibly remove much of the SDS adsorbed to the particles. After nanoparticle preparation, hydrolysis was done to ensure many carboxyl groups on the surface.

The particles were hydrolyzed in 0,5 M NaOH for 3-24 hours and the reaction stopped by centrifugation and redispergation in pure water (for ultracentrifugation
studies) or dilution with subsequent neutralization with HCl (for ultrafiltration studies).

Zeta potential as a function of hydrolysis time was studied by hydrolyzing samples for 3, 6, 18 and 24 hours.

### 4.4 Purification

Tangential flow filtration is compared to the more commonly used ultracentrifugation, with respect to purification efficiency and the stability of purified nanoparticles. The SDS content of the supernatant liquid (after centrifugation), filtrate (after filtration) and other solutions of interest was evaluated with conductometry and tensiometry. The conductometer used was a Radiometer analytical IONcheck 30 with a two-electrode dip probe. A Sigma 70 tensiometer from KSV was employed for tensiometry measurements. FTIR spectroscopy was used to study the SDS content on the purified particles.

After purification, the non-ionic surfactant Triton X-100 was added to some samples to study if it caused any SDS to leave the particles.

#### 4.4.1 Ultracentrifugation

The particles were centrifuged with a Beckman ultracentrifuge at 18000 RPM at room temperature and redispersed in distilled water on a magnetic stirrer. Occasionally, an ultrasound bath was used to aid in redispersion. Centrifugation times of 10 and 15 minutes were compared with respect to particle size and redispersion ability.

- Suspensions of 90 mg nanoparticles per ml (10 ml in 30 ml water) were centrifuged to study changes in zetapotential and size distribution.
- Suspensions of 0.9 mg nanoparticles per ml (20 ml) were centrifuged for comparison of SDS removal with filtrated samples of the same concentration.

Ultracentrifugation was also used to remove excess reagents after protein immobilization on particles (90 mg NP/ml and 0.9 mg NP/ml).
4.4.2 **Ultrafiltration**

For tangential flow filtration, the filtration module Pellicon XL from Millipore was used. The fitted membrane, UltraceL 300D, is made out of regenerated cellulose and has a MWCO (see 2.3.2) of 300 kiloDalton.

A peristaltic Masterflex pump from Cole-Parmer was used in the filtration setup. Two pressure gauges (0-60 psi) from Millipore were placed before and after the membrane, as shown in Figure 14.

**4.4.2.1 Operation and regeneration of filtration equipment**

Before filtration, the module was always flushed with clean water for approximately 30 min. After processing, it was cleaned by circulation of water for 30 minutes, 0,1 M NaOH for at least 30 minutes and subsequent flushing with a large volume of water. It was stored flat, filled with 0,05 M NaOH.

**4.4.2.2 Optimization of process conditions**

Some key parameters were determined before filtration of samples. To get the optimal process parameters, the flux through the membrane was studied at different feed flows and transmembrane pressures (TMP). This was done while recirculating the entire sample, as not to change the concentration. The feed flow was held constant while changing the TMP by restricting the retentate flow (see Figure 6). The flux through the membrane (filtrate flow per m² membrane) was plotted against the TMP for three different feed flows. The resulting curves give an indication of which flow and TMP that is appropriate in a filtration process. The optimum settings will change if different concentrations are used, as fouling occurs easier with higher concentrations [17]. Therefore, optimization was done with all concentrations used in the study; 0,45, 0,9 and 9 mg NP/ml.
4.4.2.3 Diafiltration

All filtration was done in discontinuous diafiltration mode (see 2.3.2), adding new buffer after every half DV (diavolume). 20 ml of filtrate was sampled at the end of each DV and analyzed with conductometry and tensiometry. For filtration setup, see Figure 14.

![Diafiltration Setup Diagram](image)

**Figure 14:** *Schematic figure of diafiltration setup.*

Diafiltration was done with the following solutions and settings:

- Solutions containing 0.45 mg nanoparticles per ml were filtrated at a feed flow of 40 ml/min and TMP of 11 psi, giving a flux of ~220 l/h×m² (see Figure 22). Filtration was done keeping the sample at 20°C and at 40°C, respectively, to study temperature influence on SDS removal rate. Higher temperatures were not viable because of membrane limitations.

- Solutions containing 0.9 mg NP/ml were filtrated at a feed flow of 40 ml/min and a TMP of 12 psi, giving a flux of ~200 l/h×m² (see Figure 23).

- Solutions containing 9.0 mg NP/ml were not filtrated (see 5.3.3)

4.5 Surface modification

To achieve active targeting, antibodies can be attached to a particle surface. In this project, bovine serum albumin (BSA) is used as a model protein. EDC and sulfo-NHS activate hydroxyl groups present on the particle surface, which in turn create amide
bonds with amines in the proteins. EDC is carboxyl reactive at pH 4.7-6.0 [27]. Therefore, a study of protein content after reaction at different pHs is performed.

Nanoparticles with a diameter of ~200 nm have a surface area of approximately 25 m²/g, given an estimated density of 1,19 g/cm³ (see Equation 1). For a final albumin coverage of 2-5 mg/m², concentrations corresponding to 20 and 11 mg/m² (see below) were used in the experiments.

**Equation 1**: Geometry and particle density give surface area per weight

\[
\frac{A}{m} = \frac{A}{4\pi r^2} = \frac{3}{4\pi r^3} \times \rho
\]

For the coupling reaction, the three different buffers used were phosphate buffer (0,1 M, pH 7,2) and acetate buffer (0,1 M, pH 4,0 and 5,2). Albumin was solved in buffer and added to a suspension of purified nanoparticles. Sulfo-NHS was solved in buffer and EDC was added. The sulfo-NHS/EDC-solution was added to the nanoparticle suspension which was kept at 10°C. The samples were allowed to react at room temperature overnight. Excess reagent was washed out by centrifugation and redispergation in appropriate buffer (2 x 20 ml), followed by pure water (1 x 20 ml and 1 x 10 ml). The smaller volume in the last redispergation was for doubling the nanoparticle concentration before drying and ninhydrin staining.

Immobilization of albumin on nanoparticles was done with two different nanoparticle suspensions:

- 90 mg NP/ml, purified by 5 centrifugations. 20 mg albumin/m² NP added to nanoparticles. Reaction at pH 7,2. Studied after drying by FTIR spectroscopy.
- 0,9 mg NP/ml, purified by 9DV of ultrafiltration. 11 mg albumin/m² NP added to nanoparticles. Reaction at pH 7,2/5,2/4,0. Stained with ninhydrin solution and studied by UV/vis spectroscopy.
To determine the amount of unspecific binding of BSA to the particles, the same procedure was also done with phosphate buffer instead of EDC + sulfo-NHS. This was done with a concentration of 0.9 mg NP/ml at pH 7.2.

4.6 Determination of protein content

For particle concentrations of 90 mg NP/ml, immobilization of BSA was studied with FTIR spectroscopy (4000-650 cm⁻¹). Small samples were taken after each washing step, for drying followed by FTIR analysis.

For particle concentrations of 0.9 mg/ml, the amount of primary amines on the particles, which corresponds to the amount of BSA immobilized, was determined by ninhydrin staining. After purification, 1 ml of nanoparticle suspension was mixed with 1 ml of ninhydrin solution. As a reference, 1 ml water was mixed with 1 ml of ninhydrin solution. The test tubes were placed in boiling water for 15 minutes and then in ice water for 15 minutes. 5 ml of 48% ethanol was added to each sample. After staining, the product (Ruhemann’s purple, see Figure 12) is in solutions containing primary amines. Before analysis with UV/vis spectroscopy, the nanoparticles were centrifuged down as not to disturb the measurements. A standard curve of BSA in water was made to be able to quantify the protein content with UV/vis spectroscopy.
5 Results and discussion

5.1 Preparation of nanoparticles

At SINTEF, previous preparation of nanoparticles using the miniemulsion method with MAAH and EGDMA monomers have resulted in nanoparticles around 200 nm. Using the same formula, two out of four batches generated acceptable size distributions (see Figure 15 and Table I). In the other two batches, particles >500 nm were present after polymerisation (see Figure 16 and Table I). Droplets were also visible to the naked eye prior to polymerisation in batches B3 and B4. A possible explanation is inadequate emulsification of some solutions. Beakers of diverse sizes were used for the different batches. The use of a beaker with a large diameter would permit the ultra turrax to be submerged to a greater extent, possibly solving the problem with large droplets in the solution.

Figure 15: Size distribution of MAAH particles by volume (Batch B2). Most particles are ~200 nm.
Figure 16: Size distribution of MAAH particles by volume (Batch B3). Many large particles (~500 nm) present, as well as some around 5000 nm.

Comparison of Figure 16 and Figure 17 show that filtration of batch B3 through glass wool successfully removed particles ~5000nm but, as expected, not particles ~500 nm.

Figure 17: Size distribution by volume of batch B3 after filtration through glass wool. Particles around 5000 nm are no longer present.

The zeta potentials of the different batches are totally random after polymerisation. Individual, unintentional, differences in preparation conditions result in different amounts of negative charges on the particle surfaces. Particles with low zeta potentials tend to aggregate after storage (see B2, Table I). The aggregates are insoluble by stirring or ultra sonication.
Results and discussion

Table I: Physical properties of the four batches of nanoparticles

<table>
<thead>
<tr>
<th></th>
<th>Diameter before polymerisation (nm)</th>
<th>Diameter after polymerisation (nm)</th>
<th>Zeta potential (mV)</th>
<th>Diameter 1 month storage (nm)</th>
<th>Dry material (w/w %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>179</td>
<td>208</td>
<td>-67</td>
<td>-</td>
<td>17,0 ± 0,0</td>
</tr>
<tr>
<td>B2</td>
<td>224</td>
<td>216</td>
<td>-20</td>
<td>456</td>
<td>18,1 ± 0,0</td>
</tr>
<tr>
<td>B3</td>
<td>235</td>
<td>255</td>
<td>-40</td>
<td>257 / 2000</td>
<td>17,7 ± 0,1</td>
</tr>
<tr>
<td>B4</td>
<td>234</td>
<td>954</td>
<td>-</td>
<td>-</td>
<td>16,6 ± 0,0</td>
</tr>
</tbody>
</table>

The amount of dry material is comparable in the four batches. Batch 3, which is the one used for filtration and some centrifugation, has a w/w % of 17,7. This corresponds to an approximate nanoparticle content of 177 mg NP/ml solution, regarded as 180 mg/ml in all experiments.

Only batches that showed a size distribution around 200 nm and a high initial zeta potential (Batch B1 and Batch B3 after filtration) were used in experiments.
5.2 Hydrolysis

No significant increase of nanoparticle zeta potential with hydrolysis time is detected (see Figure 18).

![Zeta potential after hydrolysis and wash](image)

**Figure 18:** Effect of hydrolysis time on nanoparticle zeta potential. Wash nr 0 shows zeta potential of nanoparticles before hydrolysis. No increase of zeta potential with hydrolysis time is seen.

The slight decrease in zeta potential after several washes indicates that negatively charged SDS is washed away. Surface hydrolysis, which is what gives the zeta potential, is evidently completed after as little as three hours. Further hydrolysis creates carboxyl groups within the particles instead.

![Hydrolysis of the particles give negative surface groups that repel the anionic surfactant SDS](image)

**Figure 19:** Hydrolysis of the particles give negative surface groups that repel the anionic surfactant SDS.
Both surface carboxyl groups and SDS molecules adsorbed to the particles give a negative zeta potential. It could be that the measured zeta potential is mainly due to SDS instead of carboxyl groups on particles hydrolyzed for only 3 hours, but surface tensiometry measurements contradict this hypothesis. When measuring on supernatant liquids, the surface tension, and thereby SDS content, is comparable for nanoparticles hydrolyzed for 3 and 24 hours, respectively (see Figure 20). Apparently, it is not only SDS giving the zeta potential on particles hydrolyzed for a shorter time, but carboxyl groups.

![Surface tension of supernatant liquid](image)

**Figure 20:** Surface tension of supernatant liquid after centrifugation. Evidently, the same amount of SDS is present on nanoparticles hydrolyzed for 3 and 24 hours.

### 5.3 Purification

Most filtration and centrifugation experiments were done only once and this affects the statistical certainty of all results. Filtration of 0.9 mg NP/ml was done with three samples to study the reproducibility of the purification process. The small variations in conductivity measurements of resulting filtrates and particle solutions show that the reproducibility is good.

#### 5.3.1 Choice of purification method and equipment

Several techniques apart from tangential flow filtration were discussed as potential purification methods, among others micro electrophoresis and the use of magnetic
beads to capture the particles. Micro electrophoresis has been used for separation in earlier studies, but it is not applicable in large scale production, i.e. in industry, which is an important criterion in this project [6]. Magnetic beads could probably be used to capture the nanoparticles, for subsequent separation from the buffer and then from the magnetic particles. Although perhaps feasible, it was believed complicated and therefore abandoned in favor of ultrafiltration. Literature studies showed that ultrafiltration is a potential candidate for nanoparticle purification, owing to its relatively efficient washing capacity, as well as being easy to scale-up and implement in industrial processes. After extensive research on the area, equipment was ordered and tested out.

Tangential flow filtration unit operations are intended for clarifying, concentrating and purifying proteins. All technical briefs and instructions available from the manufacturer consequently deal with optimization of protein purification processes, and are not always applicable on nanoparticle purification processes. The choice of equipment and processing parameters was a major part of this project and was based on reasoning, consultation of papers from previous studies ([9],[10]), as well as discussions with ultrafiltration experts at Millipore.

Choosing the right ultrafiltration equipment from the start increases the chance for success and possible scale up. The membrane material must be chosen to suit the application in question, and the membrane pore size is important to ensure high purification ability without significant loss of nanoparticles. In this project, a successful filtration process was defined by a high product yield, quality and purity. Nanoparticles of all sizes should be left in the retentate, which also should be free from aggregates and SDS. The filtrate should have a high concentration of SDS and be free from nanoparticles. The process time is important, but even more so, product yield and purity.

The filtration module chosen, Pellicon XL, is Millipore’s smallest module with a 50 cm² membrane surface, intended for process volumes from 15-1000 ml. It is part of a range of filtration modules in different sizes, enabling a linear scalability up to 10,000 L. This was an important aspect when choosing the equipment. The module is
Results and discussion

Membranes from Millipore are available in two main categories; polyethylene and regenerated cellulose. Both membranes boast low protein binding and good regeneration ability. The Ultracel cellulose membrane was chosen despite its lower pH tolerance, due to its higher compatibility with organic solvents. Organic solvents are not used in this study, but might be used for the same type of particles in the future.

The pore size (MWCO, molecular weight cut off) should be three to six times smaller than the protein to be retained [26]. A MWCO is not applicable to particles as they have no molecular weight, but approximations of the pore sizes (after measurements with scanning electron microscope) are available. The nanoparticles in this study are 100-250 nm in diameter, and the membrane chosen has an approximate pore size of 30 nm [26].

5.3.2 Operation and regeneration of filtration equipment

Both continuous and discontinuous diafiltration can be scaled up, but discontinuous was regarded easier for this lab scale study, as it does not require an extra pump. Continuous diafiltration is gentler to the product and supposedly gives a better purification [17], which is something that should be noted for further studies. In discontinuous filtration, buffer can be added before or after concentrating the sample. It was here decided to dilute the solution after concentration. This procedure requires less buffer, but might slow down the process because of an increase in the viscosity of the sample. However, no decrease in flux was noted during the process, for any of the concentrations used.

During and after filtration, there were no signs of a fouled or damaged membrane. The pressure was nearly constant throughout all filtration processes, indicating no build up of particles on the membrane. On the other hand, when flushing after filtration, the waste water was usually turbid. Particles apparently did collect within the filtration module, but never causing any constriction.
5.3.3 Optimization of ultrafiltration parameters

The study of the process parameters begins with plotting flux through the membrane against the transmembrane pressure (TMP) (for details, see 2.3.2 and 4.4.2.2). This generates an optimization curve, which gives valuable information. The settings that should give the most efficient purification can be chosen for filtration. Fouling of the membrane can also be avoided.

A high crossflow (from feed inlet to retention outlet) will reduce the concentration gradient at the membrane, giving lower risk of fouling, but also increase the retention of most components. It also leads to more passes through the membrane, which can be stressful to sensitive products. The nanoparticles are not as sensitive to mechanical stress as are proteins, and many passes through the membrane is therefore not necessarily a problem. A high TMP, on the other hand, could cause caking and fouling of the membrane, which would severely damage the process.

The knee of an optimization curve (see Figure 21) indicates where the filtrate production becomes pressure independent. Higher pressure will not increase the filtrate flow, since a membrane cake is starting to build up. For concentration filtration, the TMP and flux at the knee should be chosen. Diafiltration should be done at a TMP and flux just below the knee. This is where highest flux is achieved without exerting excessive pressure or reaching exceedingly high particle wall concentrations. At the knee, there will be polarization at the membrane and particles will begin to foul the membrane.[17]
Figure 21: Typical appearance of an optimization curve. The TMP is plotted against the flux. The knee of the curve indicates the optimum operating point. With courtesy of Millipore Inc. USA [17].

The optimum feed flow, TMP and flux through the membrane was found for each concentration of interest.
5.3.3.1 Optimization with 0,45 mg NP/ml

Optimization runs with 0,45 mg NP/ml give results as shown in Figure 22.

![Flux as function of TMP for 0,45 mg NP/ml](image)

**Figure 22:** Optimization graph for a concentration of 0,45 mg nanoparticles per ml at three different feed flows.

The membrane chosen obviously is highly permeable at low concentrations and does not easily give high TMP:s. No curvature means that no polarization occurs and that higher concentrations probably can be used. The small curvature at 30 ml/min is not due to polarization and fouling but to an almost complete restriction of the valve at the retention outlet. Almost all of the solution (25 ml/min) passes through the membrane, which consequently is not fouled.

For filtration with 0,45 mg NP/ml, a TMP of 11 psi and a feed flow of 40 ml/min was chosen. These settings give a crossflow of 20 ml/min. A higher feed flow and TMP could be used, but the process is quick enough at these settings and it will be gentler to the product and equipment.
5.3.3.2 Optimization with 0,9 mg NP/ml

Optimization runs with 0,9 mg NP/ml give results as shown in Figure 23.

**Figure 23:** Optimization graph for a concentration of 0,9 mg nanoparticles per ml at three different feed flows. Chosen filtration settings (at 40 ml/min) is marked with a circle.

With 0,9 mg NP/ml, polarization of the membrane occurs at high TMPs. A flow of 40 ml/min at a TMP of 12 psi, which is just below the knee, is used in filtration (marked with a circle). Once again, a higher feed flow could give a faster process, but the settings chosen give a lower TMP and also enable comparison with other studies.
5.3.3.3 Optimization with 9,0 mg NP/ml
Optimization runs with nanoparticle solution of 9,0 mg/ml were started, but the pressure independent region, where a higher pressure does not give a higher flux, was reached instantly. This means that the concentration of nanoparticles on the membrane surface is high and fouling of the membrane would probably occur if filtration was carried out.

5.3.4 SDS removal
Ultracentrifugation and tangential flow filtration were used to remove SDS from the particles. The SDS content of nanoparticles was analyzed with FTIR, whereas conductometry and tensiometry were used to study the SDS content of solutions. Detailed descriptions of conductometry and tensiometry as SDS quantification methods are available in chapter 5.4.

In the FTIR spectra, the peak at ~1080 cm\(^{-1}\) is interpreted as the sulfonate ion in SDS. It is present in spectra of nanoparticles after polymerization, but less evident in the spectra of hydrolyzed and washed nanoparticles (see Figure 24). This indicates a removal of SDS from particles at hydrolysis and washing. The spectra are difficult to interpret and have weak bands, decreasing the significance of these results.
Results and discussion

Figure 24: A – The sulfonate peak at 1080 cm⁻¹ is present in the spectrum of newly polymerized nanoparticles, but much less obvious after hydrolysis and two and four centrifugations, respectively. B – After five centrifugations, the sulfonate peak is not distinguishable.

Conductometry was studied as a possible quantification method of SDS in supernatant liquids and filtrates. It did not prove useful, but instead showed that the conductivity of supernatant liquids is mainly due to molecules other than SDS. (For more details,
The conductivity of filtrates and supernatants do not approach 0.8 µS/cm (conductance of pure water) at purification, but levels out at a conductance corresponding to SDS concentrations above the amount of SDS in the polymerization reaction (see Figure 27). One explanation to the high conductivity is that the small particles remaining in the supernatant liquid give conductance. This is contradicted by results seen in Figure 25. The conductivity actually increases after removal of most particles by centrifugation. This behavior could be explained by particles interfering with conductometry measurements. The negatively charged particles are relatively big and probably do not move quickly, thus not contributing to a higher conductance. Nevertheless, the negative charges might attract and thereby slow down positively charged ions, which might lead to a reduction of the conductivity. The theory of particles causing conductance in supernatant liquids is also contradicted by a corresponding conductivity of filtrates, which have very few particles in them (see 5.3.6).

**Figure 25:** Conductivity of particle solutions and corresponding supernatant liquids, before and after filtration. The conductivity increases when the particles are removed from solution.

Another explanation to the relatively high conductivity of supernatant liquids and filtrates could be that hydrolyzed oligomers of MAAH diffuse out of the particles. This is a very slow process, since they have to diffuse through a cross-linked network.
Further hydrolysis in water also occurs to some extent, which means new oligomers will diffuse out, regardless of the number of washing cycles. This theory would explain why the conductance of the supernatant liquid after centrifugation of a nanoparticle solution that was stored for one month, is higher than for the previous centrifugation (see Figure 26). The same tendency can be seen in tensiometry measurements. It is unclear if the oligomers affect the surface tension, but the surface tension decrease could be due to SDS molecules being entwined in the polymer matrix after polymerization, and released as MAAH polymers disintegrate.

![Figure 26: Tensiometry and conductometry measurements of supernatant liquids after centrifugation. The sample was stored 1 month before centrifugation number 6.](image)

The leakage of charged oligomers out of the matrix may affect the particle behavior and is not desired. There are a number of approaches to this problem:

- Use of a polymer that does not create oligomers with carboxyl groups when hydrolyzed
- Freeze drying of particles for storage to avoid further hydrolysis
- Primary hydrolysis of the particles for a shorter period of time to prevent as much hydrolysis within the particles
- Use of more cross linking agent (EGDMA) to keep the oligomers stuck in the polymer matrix
- Washing with an organic solvent to remove free polymers/oligomers from the polymer matrix

The non-ionic surfactants Mowiol, Tween 20 and Triton 100-X were used to evaluate if there were any SDS molecules adsorbed to the particles after purification. If SDS is
still adsorbed to the particles after purification, the added surfactant should replace some of it due to equilibrium, thereby causing an increase in conductivity. Despite their non-ionic structures, all of the surfactants used actually increased the conductance of pure water. Triton X-100 had the smallest influence, and was added to purified nanoparticle solutions. A small increase in conductance was noted, but measurements on solutions with hydrolyzed, non-purified particles, actually showed a slight decrease in conductance. Three ways of interpreting these results were found. Either there is no SDS on the particles after hydrolysis, purified or not, or maybe SDS has a much greater affinity to the particles than do Triton 100-X. It could also be that the method was completely useless because of the added surfactant interfering with conductometry measurements.

Since the amount of SDS can not be determined with any of the analytical methods available, a purified particle solution is therefore henceforth defined as when the conductance of filtrate/supernatant liquid doesn’t change. This gives no information on how much of the SDS is still present on the particles, but it allows comparison between ultrafiltration and centrifugation.

Centrifugation of particle concentrations of 90 mg NP/ml was preceded with dilution 1:4 in water. This gives an actual concentration of 22.5 mg NP/ml. When the samples are purified by centrifugation, it takes 4 redispergations in clean water before the conductance is stabilized, compared to 2 centrifugations with 0.9 mg NP/ml. Since all conducting acetate buffer, which was used in polymerization, probably is washed away in the first centrifugation, the decrease in subsequent centrifugations might be caused by SDS removal from the particles. The difference in SDS removal rate for nanoparticle concentrations of 0.9 and 90 mg NP/ml can be explained by the amount of buffer exchange. For the two concentrations used, the total amount of 18 mg NP washed in 7×20 ml water can be compared to 900 mg NP washed in 7×40 ml water. The buffer exchange is 25 times higher for the smaller amount, and the faster removal of SDS could be expected. Nevertheless, these measurements indicate that SDS actually is present on hydrolyzed particles, and must be washed away. The difference in conductance after it has reached a stable value can be explained by the different amounts of nanoparticles present. If the conductance is due to charged oligomers that
leak out of the particles, there will naturally be a higher conductance in a sample with more nanoparticles.

Figure 27: Conductance of supernatant liquids after centrifugation of nanoparticles of two different concentrations. The conductance reaches a stable value after few centrifugations, the number depending on the nanoparticle concentration.

Concentrations of 0,9 mg NP/ml and 0,45 mg NP/ml were filtrated. Analysis of the filtrates show that the conductance is proportional to the amount of particles filtrated (see Figure 28). This fits well with the proposed theory that it is carboxylated oligomers escaping from the particles, in addition to SDS, that give conductance.
Figure 28: Conductivity of filtrates after diafiltration. The conductivity evidently is proportional to the nanoparticle concentration.

Both diafiltration (9 DV) and centrifugation (7 runs) of solutions with 0,9 mg NP/ml give a final conductance of around 10 µS/cm in supernatant liquid/filtrate (see Figure 29). The conductance of the seventh supernatant liquid does drop as low as 8,2 µS/cm, but as the seventh wash was performed on one sample only, the measurement has no statistical certainty and should not be given too much significance. Ultrafiltration and centrifugation are therefore comparable in their ability to remove impurities in nanoparticle solutions of low concentrations.
The conductance reaches a stable value after few centrifugation or filtration cycles, the number depending on the amount of particles. The SDS is either removed at this stage, or adsorbed strongly to the particles. Previous studies at SINTEF show that there is little or no adsorption of protein to particles covered with SDS. As there is adsorption of BSA to the particles (see 5.5), this indicates a successful removal of SDS from the particles with centrifugation as well as with ultrafiltration.

A temperature dependent critical micelle concentration for SDS (Berthod, 2000) indicates that the solubility is higher at higher temperatures. If more SDS is dissolved instead of adsorbed to the particles, it should lead to a larger amount of SDS in the filtrates and thereby a more efficient purification. To study differences in SDS removal rates, solutions of 0.45 mg NP/ml were filtrated at 20° and 40° C, respectively. The conductivities of filtrates after filtration at 40° C were slightly higher than for the ones after filtration at room temperature (see Figure 30), but the effect is not significant enough to recommend elevated temperatures.
Figure 30: No significant effect of temperature on SDS concentration in filtrates after filtration of nanoparticle solutions of 0.45 mg NP/ml can be seen.

5.3.5 Process time and efficacy

In both centrifugation and filtration, it is possible to purify large batches of nanoparticles at a time. The extent of buffer exchange needed for purification is nevertheless the same. Purification of more concentrated samples by centrifugation demands a larger amount of buffer extent before they can be considered clean. Process time in centrifugation therefore depends on concentration of sample and size of centrifugation vessel. In filtration, there is no such delimiting factor. Larger process volumes result in longer process times, but the same equipment can be used. The membrane area can be increased to handle higher concentrations, making the unit mg NP/cm² membrane important in filtration studies.

The concentrations and settings used in this study are:

- 0.45 mg NP/ml – crossflow of ~20 ml/min and flux ~220 l/h×m².
- 0.90 mg NP/ml – crossflow of ~23 ml/min and flux ~200 l/h×m².

The flux is lower for higher concentrations because of higher viscosity of the sample. When increasing the concentration of nanoparticles to make the purification process
faster, this decrease in flux must be considered. Too high concentrations will actually make the process slower, and, in the worst case scenario, foul the membrane. This can be prevented by doing optimization runs before processing.

Filtration of 0.45 mg NP/ml is not interesting when discussing process times, as filtration with higher concentrations was feasible. Concentrations of 0.9 mg NP/ml were filtrated without any signs of fouling of the membrane. Even higher concentrations might very well be possible to filtrate with the membrane used. However, a concentration of 9.0 mg NP/ml is too high.

Comparison between ultrafiltration and centrifugation is done with 0.9 mg NP/ml. When comparing purification efficacy, the nanoparticles are considered clean when the conductance of supernatant liquid/filtrate has leveled out at 10 µS/cm. It is assumed that there is no SDS on the particles at this stage, and the lingering conductivity is a consequence of charged oligomers slowly leaking out from the particles.

Figure 31 illustrates the removal of SDS from a nanoparticle solution of 0.9 mg/ml, with a volume of 20 ml when centrifuging and 100 ml when filtrating. When the buffer is exchanged, soluble molecules such as SDS will follow it. Therefore, buffer exchange purifies the particles in both techniques. In diafiltration it is measured in produced diavolumes, and with a fifth of the starting volume (20 ml), the buffer exchange would go 5 times faster, given the same conditions. The centrifugation process can not be done any faster, but with larger volumes. As can be seen in Figure 31, filtration reaches a conductance of 10 µS/ml after half an hour, whereas purification by centrifugation needs 45 minutes. It should be noted though, that centrifugation has large intervals (15 minutes) and that the supernatant liquid has a conductance of 13 µS/cm after only 30 minutes. The nanoparticles that have been redispersed in pure water after two centrifugations might be pure.

No huge differences in performance for centrifugation and diafiltration can be observed as seen in Figure 31. To estimate the efficiency, one must also consider possibilities for up-scaling of the process.
Figure 31: Conductance of supernatant liquids and filtrates after purification with centrifugation and filtration, respectively. Nanoparticle concentration of 0.9 mg/ml.

With respect to the removal rate, discontinuous diafiltration is superior to centrifugation at these volumes and concentrations (see Table II). With used settings, equipment and concentration, 150 mg of nanoparticles can be purified in one hour. The use of a module with 1 m² membrane would enable purification of 30 g nanoparticles per hour. This is almost 10 times more than what centrifugation can accomplish in the vessels used, where one hour is needed to reach a stable conductivity value for 40 ml of 90 mg NP/ml (see Figure 27). Though, the use of a larger membrane is not the first thing to do to make the process more efficient. More important is the optimization of concentrations, TMP:s and fluxes. The flux used is 200 l/h×m² and there is probably room for many modifications to enhance the purification by ultrafiltration.
Results and discussion

Table II: Purification efficiency of filtration and centrifugation. 0,9 mg NP/ml. Results from conductometry measurements.

<table>
<thead>
<tr>
<th></th>
<th>Filtration (100 ml)</th>
<th>Centrifugation (20 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conductivity of initial sample</td>
<td>295 µS/cm</td>
<td>295 µS/cm</td>
</tr>
<tr>
<td>Conductivity of purified sample</td>
<td>10,0 µS/cm</td>
<td>10,0 µS/cm</td>
</tr>
<tr>
<td>Time to purify sample</td>
<td>36 min</td>
<td>45 min</td>
</tr>
<tr>
<td>Time to purify 20 ml</td>
<td>7,2 min</td>
<td>45 min</td>
</tr>
</tbody>
</table>

The crossflows used (20 and 23 ml/min respectively) were lower than recommended by the manufacturer (30-50 ml/min). In previous studies, values significantly higher and lower has been used, as seen in Table III. According to the manufacturer, too low crossflows can cause particles to get stuck in the membrane if they are small compared to the pore size, but no such fouling occurred.

Dalwadi et.al. (2005) have used very low flows throughout their study. Limayem et.al. (2004) used higher concentration and cross flow, resulting in a comparatively low and furthermore a decreasing flux, perhaps due to high viscosity and small (80 nm) nanoparticles fouling the membrane. However, both groups report a successful removal of PVA from nanoparticles. This is done by concentration filtration and/or diafiltration for over 2,5 hours, with volumes of 2000 and 200 ml, respectively. Presupposed that SDS really has been removed in this study, the process was much faster, even when taken the lower volume (100 ml) into consideration. If successful removal of surfactant from particles has been achieved with these three very different setups, there is probably much room for modifications of parameters and settings to optimize purification efficiency of tangential flow filtration.
Table III: Filtration settings and results for this study, compared to two earlier ones.

<table>
<thead>
<tr>
<th></th>
<th>Flux (l/h×m²)</th>
<th>TMP (psi)</th>
<th>Cross flow (ml/min)</th>
<th>Conc (mg/ml)</th>
<th>NP/area (mg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td>200</td>
<td>12</td>
<td>23</td>
<td>0,9</td>
<td>1,8</td>
</tr>
<tr>
<td>Dalwadi et.al. (2005)</td>
<td>13,5</td>
<td>10</td>
<td>2</td>
<td>0,5</td>
<td>2</td>
</tr>
<tr>
<td>Limayem et.al (2004)</td>
<td>20 - 3</td>
<td>14,5</td>
<td>1000-1700</td>
<td>11,5²</td>
<td>23²</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Process</th>
<th>Fouling</th>
<th>Process time (min)</th>
<th>NP size (nm)</th>
<th>Pore size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td>D</td>
<td>No</td>
<td>60</td>
<td>200</td>
<td>30</td>
</tr>
<tr>
<td>Dalwadi et.al. (2005)</td>
<td>D/C</td>
<td>No</td>
<td>170</td>
<td>-</td>
<td>30</td>
</tr>
</tbody>
</table>

D = diafiltration, C = concentration filtration, / = or, + = and, a = Reference: Dalwadi et.al (2005).

5.3.6 Yield – nanoparticles lost to filtrate/supernatant liquid

When purifying by centrifugation, nanoparticles could be lost to the supernatant liquid. With the wrong equipment and settings in filtration, nanoparticles could either stick to the membrane or flow through it and be lost to the filtrate.

Particle size measurements of filtrates after purification by diafiltration gave random number distributions between 0,6 nm and 140 nm for different measurements of the same sample. Since the correlation function did not have its normal form during these measurements, the number distribution is not very reliable (see 2.6.2). The error message “Not suitable for analysis” appeared at some measurements, as it does when measuring pure water. These results are interpreted as practically no nanoparticles lost to the filtrate. According to the membrane manufacturer Pall, the used membrane with a MWCO of 300 kD should be able to retentate 96-99% of particles in the significant size range [26]. Turbid waste water when flushing the equipment after processing, indicate that particles do collect in the filtration module, but without disturbing the process. This loss was not quantified.
Centrifugation of nanoparticle solutions of 90 mg NP/ml gave a turbid supernatant liquid. The turbidity decreased with every additional centrifugation, probably due to the finer particles being removed from the nanoparticle stock. Particle size measurements showed particles larger than 100 nm present in the supernatant liquids after 10 minutes of centrifugation, while the supernatant liquids after 15 minutes of centrifugation only contain particles between 50 and 100 nm (see Table IV). Centrifugation of 0.9 mg NP/ml did not result in a turbid supernatant, probably since there are not as many fine particles present from the beginning. Nevertheless, the loss is likely to be proportional to the loss for a higher concentration.

Table IV: Size of particles in supernatant liquid after centrifugation for 10 and 15 minutes at 18000 RPM. The loss of big nanoparticles is highest after short centrifugation.

<table>
<thead>
<tr>
<th>Number of centrifugations</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min</td>
<td>169 nm</td>
<td>120 nm</td>
<td>-</td>
<td>116 nm</td>
<td>-</td>
</tr>
<tr>
<td>15 min</td>
<td>71 nm</td>
<td>76 nm</td>
<td>92 nm</td>
<td>96 nm</td>
<td>89 nm</td>
</tr>
</tbody>
</table>

No reliable means of examining the concentration of particles was available in this study. The only way to determine the loss of nanoparticles was by weighing samples before and after purification. 1800 mg nanoparticles (10 ml) were taken from batch B1 for hydrolysis and purification. Gravimetric measurements of a sample that had been centrifuged and redispersed 5 times show a nanoparticle content of 2.75±0.05 w/w %, corresponding to only 1100 mg nanoparticles. This is a large loss of material, but most of it was probably not lost to the supernatant liquid. Between centrifugations, the nanoparticles were moved between the plastic centrifugation tubes and glass beakers to enable ultra sonication. Visible amounts were left in the beakers, as it was impossible to remove it all. The loss would be reduced significantly if it was possible to redisperse the nanoparticles in the centrifugation vessel. The loss of nanoparticles to the supernatant liquid could not be quantified.
Gravimetric measurements of filtrated samples were not feasible because of the low concentrations. Evaporation of approximately 10 ml sample resulted in a very small amount of white, light powder. It is highly probable that some of it blew away before weighing and the results are not reliable.

Ultrafiltration does not seem to be subject to loss of nanoparticles at any great extent, as opposed to centrifugation at the settings used. However, the loss of finer nanoparticles is not necessarily a problem, since the smaller particles count for a larger SDS content per volume than do the larger ones.

Turbidity measurements with for example UV/vis could perhaps assist in a more accurate determination of nanoparticle content. It was discussed as part of this project, but not performed, since there were no means of making a standard curve with known concentrations. More accurate determination of material loss in ultrafiltration would be possible if higher concentrations were filtrated.

5.3.7 Quality – stability of product

Table V summarizes the physical properties of nanoparticles after purification. Neither ultrafiltration nor centrifugation affects the particle size significantly. Even when much of the stabilizing SDS had been washed away were there any problems with irreversible aggregation, probably due to the carboxylic acid surface groups. The particles used are relatively large and have high zeta potentials. However, particles with BSA immobilized on the surface have a lower zeta potential due to the low amount of carboxyl acid surface groups. Centrifugation of these causes aggregation (see 5.5).

One third of the size measurements of filtrated particles show a small (10%) portion of nanoparticles around 5000 nm. Evidently, there are some aggregates. Particle size measurement of the initial solution showed no aggregates, but it was measured only once. The presence of aggregates in the initial solution can not be ruled out, since the volume taken for analysis is not necessarily representative for the entire sample. Filtrated samples were analyzed three times, and it is unclear if the observed aggregates were present from the beginning, or are a result of the filtration process.
Heydenreich et. al. (2003) experienced a 3-fold increase in nanoparticle size after 1 month storage of filtrated particles. All purified particles in that study had a zeta potential between 10-20 mV. As seen in Table V, no change is observed after storage of the particles purified in this study, probably owing the high zeta potential. The large deviations in size measurements are due to a small amount of samples and large size distributions. Additional analyses on different volumes would most likely decrease the deviations. The physical stability of the product was not affected by filtration at higher temperature.

Table V: Physical stability of nanoparticles after purification by different processes. Analyzed before and straight after purification, as well as 1 month after purification.

<table>
<thead>
<tr>
<th>Process</th>
<th>Size (nm)</th>
<th>Zetapotential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Diafiltration</td>
<td>211±9</td>
<td>228±12</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>246±0</td>
<td>244±15</td>
</tr>
</tbody>
</table>

5.4 Methods to determine SDS-content

Precipitates after centrifugation, as well as dried nanoparticles, were studied in an FTIR spectrometer. FTIR is not a quantitative method and could only serve as a guide when studying the change in SDS content on the particles. Insufficient contact between sample and IR crystal resulted in weak bands on the spectra made the method even less suited for SDS content determination in this study. Measurement of more material might solve this problem.

Analysis of nanoparticle solutions, filtrates and supernatants do not measure the actual SDS content of the particles. Nevertheless, since there will always be equilibrium between SDS adsorbed to the particles and free in solution, it should be possible to use solution measurements to determine the SDS content on the particles.
The analytical methods used in this project were inadequate for determination of the amount of SDS in solutions. The conductometry measurements were interfered by other charged molecules in the solutions (see 5.4.2), and the tensiometry measurements were not sensitive enough (see 5.4.1). A tensiometer with auto-dilution might handle the low concentrations in the samples better than the tensiometer used. Rusconi et. al (2001) were able to quantify SDS in biochemical samples by using a stain that changes color in the presence of SDS [14]. This method might be applicable on supernatant liquids and filtrates. Further literature studies can perhaps reveal other methods to quantify SDS, both in solutions and adsorbed on particles.

5.4.1 Tensiometry

When the critical micelle concentration is reached, the entire surface of the liquid is occupied by surfactant molecules. Additional surfactant will form micelles and therefore not affect the surface tension. The standard curve for SDS shows a well defined break below 10 mM, indicating the CMC (see Figure 33 A).

![Tensiometry](image)

**Figure 32:** Theoretical shape of surface tension as function of concentration. The break (vertical line) indicates the CMC. A standard curve of concentrations within the linear part of the curve (indicated by circle) makes quantification straightforward.

A tensiometry curve theoretically has the shape shown in Figure 32. As presented in Figure 33 A, the concentrations used in this study (~5,0 – 0,01 mM) is partly outside
the linear part of the curve (see Figure 33), making quantifications very difficult. In addition, tensiometry measurements are extremely sensitive to contaminations. It is not easy to create a standard curve over such low concentrations without automatic dilution. This is possible for some tensiometers, but not the one used. The standard curve made in this study has a low correlation coefficient $R^2$ (see Figure 33 B) and was not used for determining SDS concentrations.

**Figure 33**: Standard curves for SDS concentration in tensiometry, for all used (A) and only low (B) concentrations.

### 5.4.2 Conductometry

The Zetasizer from Malvern instruments measures conductance as part of a zetapotential measurement. This was used in an early stage as a conductometry instrument. Although, after several centrifugations the particle concentration in the supernatant liquid got too low to measure the zeta potential, and therefore it was no longer possible to get information on the conductance. Instead, the Radiometer conductometer described above was used for all measurements.
At SDS concentrations over CMC, additional surfactant will not affect the amount of free floating molecules in the solution. The micelle concentration will increase, but since micelles are so big, they do not move very fast and therefore will not contribute significantly to the conductance. The standard curve for SDS levels out at a concentration below 10 mM, indicating the critical micelle concentration (see Figure 34). This is in accordance with the literature value [37].

Figure 34: Standard curve for SDS concentration in conductometry measurements.

Centrifugation of particles at a concentration of 90 mg/ml gives a decrease in the conductance of the supernatant liquids as shown in Figure 35. The first conductance measurement of 20 000 µS/cm would not be possible to achieve with only SDS molecules conducting the current, as it would require SDS concentrations extremely higher than the ones used in the study. The SDS concentration in the polymerization reaction is approximately 11 mM, corresponding to a maximum conductance of 600 µS/cm, as seen in Figure 34. Higher conductance values must therefore be due to other molecules in the solutions. The acetate buffer present after polymerization has a high conductance, but as it is not adsorbed to the particles, it should be washed away in the first centrifugation. This is the case also for NaOH which was added to hydrolyze the polymers, although pH measurements show that most of the OH⁻ ions are consumed during hydrolysis. Particles with surface charges are also present to a
certain extent in each supernatant liquid, but according to measurements, these tend to decrease rather than increase the conductance (see Figure 25).

After 4 centrifugations, the conductance is stable at 50 µS/cm, or at least only decreasing slowly (see Figure 35). 50 µS/cm corresponds to an SDS concentration of approximately 0,75 mM. A concentration of 0,75 mM SDS in each supernatant liquid adds up to a value above the initial SDS concentration. It is therefore highly unlikely that SDS is the only molecule contributing to conductance once acetate buffer and NaOH have been washed away.

![Conductance of supernatant liquids](image)

**Figure 35:** Conductance of supernatant liquids after purification by centrifugation of solution containing 90 mg NP/ml.

The same pattern is observed when measuring the conductivity of supernatant liquids after centrifugation of solutions with 0,9 mg nanoparticles/ml (see Figure 36). The conductivity reaches a stable value after only one centrifugation. This value corresponds to an approximate SDS concentration of 0,1 mM. Hydrolysis of a nanoparticle suspension with subsequent dilution to 0,9 mg NP/ml, gives a maximum total SDS concentration of 0,05 mM. It is therefore obvious that the conductivity is mainly due to charged molecules other than SDS.
Conductance of supernatant liquids

![Graph showing conductance of supernatant liquids after purification by centrifugation of solution containing 0.9 mg NP/ml.](image)

**Figure 36**: Conductance of supernatant liquids after purification by centrifugation of solution containing 0.9 mg NP/ml.

Conductometry is not a selective method, and apparently there are conducting molecules other than SDS present in the solutions. This makes conductometry unsuitable for SDS quantification in this project. If one was sure that there were no other disturbing molecules in the solutions, conductometry measurements could be used online in TFF to determine when a high enough level of purity has been reached.

### 5.5 Protein immobilization

Protein immobilization was done on particles purified by 5 centrifugations (90 mg NP/ml) and on particles purified by diafiltration for 1 hour (0.9 mg NP/ml). Surface carboxyl groups were activated with EDC/NHS and chemically coupled to bovine serum albumin.

Aggregation of particles seems to happen during the immobilization reaction (see Table VI). Since the zeta potential does not change during washing, the observed decrease in size is probably due to particle aggregates being dissolved, rather than loss of surface adsorbed molecules. If the proteins had cross-linked the particles by forming amide bonds with more than one particle at a time, the aggregates would be insoluble. As sonication dissolves the aggregates, aggregation is more likely caused by a low zeta potential. Particles with zeta potentials below ±30 mV tend to aggregate...
(see 2.6.1). It is assumed that proteins attached to the particle surfaces affect the zeta potential. The negatively charged carboxyl groups that gave a high zeta potential to unmodified particles, have now formed amide bonds with proteins. The proteins expose negative charges to the surroundings, but not enough to create the high zeta potential needed for a stable system. It is likely, therefore, that there are proteins attached to the particle surfaces, and that the particle solutions need constant stirring and ultra sonication after centrifugation to avoid aggregation of particles.

Table VI: Physical properties of nanoparticles during immobilization of proteins and subsequent washing procedure (90 mg NP/ml, immobilization reaction at pH 7.2, washed by centrifugation). The zeta potential decreases after immobilization of BSA.

<table>
<thead>
<tr>
<th></th>
<th>Size (Vol. dist.) (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial sample</td>
<td>222</td>
<td>-44</td>
</tr>
<tr>
<td>After mixing with reagents</td>
<td>265</td>
<td>-25</td>
</tr>
<tr>
<td>Before purification, in buffer</td>
<td>492/5111</td>
<td>-19</td>
</tr>
<tr>
<td>After 1st wash</td>
<td>291/4972</td>
<td>-23</td>
</tr>
<tr>
<td>After sonication</td>
<td>377</td>
<td>-23</td>
</tr>
<tr>
<td>After 2nd wash, sonicated</td>
<td>305</td>
<td>-22</td>
</tr>
<tr>
<td>After 3rd wash, sonicated</td>
<td>295</td>
<td>-22</td>
</tr>
<tr>
<td>After 4th wash, sonicated</td>
<td>276</td>
<td>-21</td>
</tr>
</tbody>
</table>

FTIR was used to study particles before and after BSA immobilization (see Figure 37). The spectra are of poor quality, probably due to insufficient contact between the sample and the instrument crystal. Several attempts were made, but no acceptable spectra were obtained. The use of more material might solve this problem. The spectra do not give any clear results, but a decrease in the peak interpreted as a carboxyl group (~1560 cm\(^{-1}\), solid circle) indicates that a reaction has taken place on the surface. Small indistinct changes in the areas where amide bonds should be seen in the spectrum for particles after immobilization reaction (1640 cm\(^{-1}\) and 1520 cm\(^{-1}\), dashed circles) point to some immobilized proteins on the surface, but no large amounts.
Figure 37: FTIR spectra of nanoparticles without and with immobilized BSA. The solid circle marks the peak interpreted as carboxyl group. The dashed circles mark areas where amide bonds should be seen.

The pH during the immobilization reaction will affect several factors:

- BSA has a pI of approximately 5.0 [24]. At pH 4.0 there are not as many negative charges on the protein as there are at pH 7.2 and 5.2.
- EDC is carboxyl reactive at pH 4.7-6.0 [27]. Activation of carboxyl groups, for subsequent reaction with proteins, should therefore work best at pH 5.2, out of the pH:s used.
- Carboxyl groups have a pI around 4.0 [24]. There are less negative charges on the particle surfaces at a low pH.

A low pH will reduce the amount of negative charges on particles and BSA, thereby decreasing the electrostatic repulsion. Activation of carboxyl groups seems to work at pH 7.2 (see immobilization results in Table VI) but it might be more efficient at pH 5.2, which is the only used pH within the optimum interval for EDC.
Ninhydrin staining of the samples led to color changes which were detected by UV/vis spectroscopy. Estimation of the BSA concentrations was done using a standard curve (Appendix C). The color change is proportional to the amount of BSA on the particles and gives no information on the nature of the bond. The proteins can be either adsorbed to the particles, or covalently bound. A covalent bond between protein and nanoparticle is necessary. The proteins could otherwise diffuse out to the blood when within the body, and be replaced with other random proteins. Addition of BSA to the nanoparticles without activation of the carboxyl groups with EDC, shows that there is adsorption of BSA to the particles (see Figure 38). This control was done at pH 7,2. It is likely that there is even more adsorption of BSA on nanoparticles in more acidic environments, as there is lower electrostatic repulsion. Activation of carboxyl groups before the addition of protein, and not the reverse as was done in this study, could possibly prevent some of the unspecific adsorption.

![Amount BSA immobilized on nanoparticles](image)

**Figure 38:** Amount BSA immobilized on nanoparticles at different pH and without activation of carboxyl group with EDC/NHS as control. A NP-concentration of 0,9 mg NP/ml is assumed.

No accurate method to determine nanoparticle concentrations has been available in this project. The nanoparticle loss after filtration, immobilization and subsequent centrifugation is therefore not known. Gravimetric measurements before filtration gave an approximate concentration of 0,9 mg NP/ml. Assuming there was no loss during the process, 2,6 µmol BSA can be immobilized on 1,0 g of nanoparticles, as
seen in Figure 38. This assumption is most likely an overestimation of the nanoparticle content. If half of the nanoparticles were lost to the membrane in filtration and to the supernatant liquid in centrifugation, the BSA content consequently is the double.

Ninhydrin staining gives no information on how much of the protein content that is covalently bound to the particles, and how much that is only adsorbed. The level of adsorption might depend on the pH and the only estimate that can be given is for the reaction done at pH 7,2, at which a control immobilization was done without EDC/NHS. 0,41 µmol more protein is immobilized per gram nanoparticles when the carboxyl groups are activated, than when no activation is performed. This is regarded as the minimal amount of covalently bound protein. It is highly likely that a larger amount is covalently bound when the carboxyl groups are activated, but this is not possible to read out from the results obtained. To study the extent of covalent immobilization, the particles could be washed with SDS after the immobilization reaction. This would remove any adsorbed protein and allow subsequent quantification of covalently bound proteins by ninhydrin staining.

A minimum value of 0,41 µmol covalently bound BSA/g NP, for an assumed nanoparticle concentration of 0,9 mg NP/ml, corresponds to a surface coverage of 1,1 mg BSA/m². For the same assumed nanoparticle concentration, the highest level of immobilization measured (achieved at pH 4,0) was 6,8 mg BSA/m², whereof an unknown part is covalently bound to the particles. This value is high above the expected level of 2-5 mg BSA/m² NP.

BSA has an ellipsoid shape and an approximate size of 4×14 nm [36]. Assuming a protein content of 2,0 µmol per gram of nanoparticles and upright proteins, this corresponds to a coverage of 76% of the nanoparticle surfaces. Absorption of proteins to the particles when no activation was performed indicates that the surface coverage of negatively charged carboxyl groups is rather low, since they otherwise would repel the proteins. It is also a clear indication of successful removal of SDS. Proteins readily adsorb to free polymer surface, and it is also possible that a second layer of proteins is formed on the first layer. Adsorbed proteins could be washed away with SDS to leave only covalently bound BSA on the particles. It is also possible that
Results and discussion

Adsorbed proteins can be shaken off by ultrasonication. This would explain the weak signals obtained in FTIR spectroscopy after immobilizing BSA on nanoparticles of concentration 90 mg/ml, as they were ultrasonicated after wash.

It was known from immobilization of protein on particles using high particle concentrations that the particles tend to aggregate during the reaction. Nevertheless, with such low concentrations as 0.9 mg NP/ml, it was not an option to move the nanoparticles from the centrifugation tubes for redispersion in another vessel as it would lead to loss of most of the material. This meant that no ultrasonication was possible, because of the centrifugation tubes being made out of plastic. Size measurements after immobilization reaction and washing by centrifugation show that there are aggregates in all samples (see Table VII). If the particles aggregated after immobilization, and not before, it is possible that the ninhydrin solution did not react with all of the BSA immobilized on the particles. The BSA content could therefore be higher than reported here.

**Table VII:** Physical properties of nanoparticles before and after immobilization reaction and wash. There is extensive aggregation after immobilization of BSA when the samples are not ultrasonicated.

<table>
<thead>
<tr>
<th>pH</th>
<th>Size (vol. dist.)</th>
<th>Aggregates present</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before reaction</td>
<td>208</td>
<td>None</td>
<td>-53</td>
</tr>
<tr>
<td>pH 7.2 – without EDC/NHS</td>
<td>400</td>
<td>5000 / 13%</td>
<td>-34</td>
</tr>
<tr>
<td>pH 4.0</td>
<td>323</td>
<td>1000 / 54%</td>
<td>-24</td>
</tr>
<tr>
<td>pH 5.2</td>
<td>4700</td>
<td>4700 / 100%</td>
<td>-37</td>
</tr>
<tr>
<td>pH 7.2</td>
<td>243</td>
<td>1700 / 98%</td>
<td>-39</td>
</tr>
</tbody>
</table>

The zeta potential after the immobilization reaction corresponds in part to the protein content. The lowest (negative) zeta potential as well as the highest BSA content is measured after coupling at pH 4.0. After reaction at pH 5.2 and 7.2, both zeta
potential and protein content are comparable. A lower BSA content corresponds to a higher negative zeta potential for all samples except the control, where no activation of the carboxyl groups was performed. The surface coverage of the control is approximately 60% and if negatively charged carboxyl groups are exposed to the surroundings, this could explain the higher negative zeta potential.
6 Conclusion

Tangential flow filtration was evaluated as an alternative purification method for nanoparticles, since it can be implemented in industrial processes. It is found to be an efficient purification method that is comparable to ultracentrifugation with respect to SDS removal. The yield is high as it does not suffer from loss of material to the filtrate, but there are indications of slight aggregation of the filtrated nanoparticles.

With the analytical methods available for this project, a definite conclusion regarding SDS removal cannot be drawn. Either the SDS is removed after a few centrifugations/diavolumes, the number depending on the amount of nanoparticles, or it is strongly adsorbed on the nanoparticles. If there is SDS adsorbed to the particles, it will be further removed during purification necessitated by surface modification. However, BSA adsorption on the particles suggests there is no SDS present, as earlier studies at SINTEF has shown that there is little or no adsorption of proteins on particles carrying SDS.

BSA is readily immobilized on the particles, but a considerable part of it is most likely adsorbed instead of covalently bound. After immobilization reaction at pH 7,2, a minimum of 1,1 mg BSA was covalently bound to 1 m² of particle surface, but this is probably an underestimation. Reaction at a lower pH gives a higher amount of protein on the particle surface. An estimation of 2,0 µmol covalently bound BSA per gram of nanoparticles corresponds to 5,3 mg/m² and a surface coverage of 76%. Protein coupling decreases the zeta potential of the particles, causing aggregation. The aggregates are soluble by ultrasonication, but this is not possible during ultrafiltration.
7 Recommendations

To ascertain the removal of SDS from the particles, other analytical methods should be tested out. XPS is a surface sensitive method that could be used to detect sulfur, and thereby SDS. FTIR results could possibly be improved if larger batches were processed. However, none of these methods is quantitative, and other alternatives should be evaluated. Rusconi et. al (2001) were able to quantify SDS in biochemical samples by using a stain that changes color in the presence of SDS [14]. This method might be applicable on supernatant liquids and filtrates.

If SDS is found difficult to remove, other stabilizers should be evaluated for use in preparation of nanoparticles for medical applications. The surfactant PVA has been washed away successfully from nanoparticles in previous ultrafiltration studies, and might be useful for stabilizing the particles in question. According to Perrachia et. al. (1997), PEG can also be used to stabilize miniemulsions [13]. Another alternative is the fibrils that are under development at SINTEF, due to their surfactant like properties and biological compatibility [15].

Since tangential flow filtration was found to be a promising alternative to centrifugation for removal of SDS from nanoparticles, it ought to be studied further. Higher concentrations could speed up the process times, as well as higher crossflows. To fully make use of the advantages with tangential flow filtration, it must be possible to purify the particles after surface modification as well as after polymerization. Proteins smaller than 67 kD will not be retained by a membrane with a MWCO of 300 kD, according to Pall ([26]). To remove other proteins, a membrane with another MWCO might be better suited. A problem that must be addressed before filtration of surface modified particles can be performed is their instability. Ultrasonication is not possible during filtration, and one must therefore ascertain that the particles do not aggregate during purification.
8 References


[17] Protein Concentration and Diafiltration by Tangential Flow Filtration, [Internet source], Millipore, Viewed 2007-10-01, Available at http://www.millipore.com/techpublications/tech1/tb032


[26] Select the Proper Separation Product for Your Application (Centrifugal, TFF), [Internet Source], Pall Instruments, Viewed 2007-10-03, Available at http://www.pall.com/laboratory_7046.asp


[28] Zeta Potential An Introduction in 30 minutes, [Internet source], Malvern Instruments, viewed 2007-09-22, Available at http://www.malvern.co.uk/LabEng/technology/zeta_potential/zeta_potential_LDE.htm (login now required for some information)

[29] Dynamic Light Scattering (DLS), [Internet source], Malvern Instruments, viewed 2007-12-04, Available at http://www.malvern.co.uk/LabEng/technology/dynamic_light_scattering/dynamic_light_scattering.htm


[31] Regina Vs Susan Hilda May, [Internet Source] Viewed 2008-01-03, Available at http://www.susanmay.co.uk/student-report2

[32] Surface tension, [Internet source], KSV Instruments, viewed 2007-10-19, Available at http://www.ksvltd.com/content/index/keytensiometer

[33] Tensiometer, [Internet source], KSV Instruments, viewed 2007-10-19, Available at http://www.ksvltd.com/content/index/keytensiometer
[34] *Conductometry*, [Internet source], Britannica Online Encyclopedia, viewed 2007-10-10, Available at http://www.britannica.com/eb/print?articleID=110403&fullarticle=true&tocId=80808


9 List of appendices

A. Chemicals
B. Buffers and ninhydrin solution
C. Standard curve for BSA concentration in UV/vis spectroscopy measurements
Appendix A

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical formula</th>
<th>Distributor</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>CH₃COOH</td>
<td>Merck</td>
<td>99.8%</td>
</tr>
<tr>
<td>Albumin, Bovine</td>
<td></td>
<td>SIGMA</td>
<td>&gt; 96 %</td>
</tr>
<tr>
<td>Dimethyl sulfoxide DMSO</td>
<td>(CH₃)₂SO</td>
<td>Merck</td>
<td>≥ 99.5 %</td>
</tr>
<tr>
<td>EDC</td>
<td>C₈H₁₂N₃</td>
<td>Fluka</td>
<td>≥ 97.0 %</td>
</tr>
<tr>
<td>EGDMA</td>
<td>C₁₀H₁₄O₄</td>
<td>Fluka</td>
<td>&gt; 90 %</td>
</tr>
<tr>
<td>Lithium hydroxide</td>
<td>LiOH</td>
<td>Merck</td>
<td>&gt; 98 %</td>
</tr>
<tr>
<td>Mowiol N 70-88</td>
<td></td>
<td>Hoechst</td>
<td></td>
</tr>
<tr>
<td>MAAH</td>
<td>[H₂C=C(CH₃)CO]₂O</td>
<td>Fluka</td>
<td>92 %</td>
</tr>
<tr>
<td>n-Hexadecane</td>
<td>C₁₆H₃₄</td>
<td>VWR Int.</td>
<td>&gt; 99 %</td>
</tr>
<tr>
<td>Ninhydrin</td>
<td>C₉H₆O₄</td>
<td>Merck</td>
<td>&gt; 99 %</td>
</tr>
<tr>
<td>Potassium Iodide</td>
<td>KI</td>
<td>Haën</td>
<td>&gt; 99.5 %</td>
</tr>
<tr>
<td>Sodium acetate trihydrate</td>
<td>CH₃COONa x 3 H₂O</td>
<td>Merck</td>
<td>99.0-101.0 %</td>
</tr>
<tr>
<td>SDS ( Sodium dihydrogen)</td>
<td></td>
<td>Merck</td>
<td>&gt; 99 %</td>
</tr>
<tr>
<td>Dodecylsulfate salt</td>
<td>C₁₂H₂₅NaO₄S</td>
<td>Merck</td>
<td>&gt; 99 %</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate dihydrate</td>
<td>NaH₂PO₄ x 2 H₂O</td>
<td>Merck</td>
<td>98.0-100.5 %</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>NaOH</td>
<td>Merck</td>
<td>98.0-100.5 %</td>
</tr>
<tr>
<td>Stannous chloride dihydrate</td>
<td>Cl₂Sn.H₂O</td>
<td>Merck</td>
<td>&gt; 98 %</td>
</tr>
<tr>
<td>sulfo-NHS</td>
<td>C₄H₄N NaO₆S</td>
<td>Fluka</td>
<td>≥ 93.5 %</td>
</tr>
<tr>
<td>Triton X-100</td>
<td></td>
<td>DOW</td>
<td></td>
</tr>
<tr>
<td>Tween 20</td>
<td></td>
<td>Fluka</td>
<td></td>
</tr>
<tr>
<td>V-65 (2,2’-Azobis(2.4-dimethyl valeronitrile)</td>
<td></td>
<td>Wako Chem.</td>
<td></td>
</tr>
</tbody>
</table>
Appendix B

Ninhydrin solution
Ninhydrin solution is prepared while constantly flushing the solution with \( \text{N}_2 \), as ninhydrin is highly sensitive to oxygen.

Dissolve 2 g of ninhydrin in 75 ml of DMSO while stirring for 30 minutes. Add 40 mg of stannous chloride dihydrate, followed by another 30 minutes of stirring. Finally, add 25 ml of lithium acetate buffer (pH 5,2). Ninhydrin solution is sensitive to light, heat and oxygen. It should be stored cold and dark and used within two days.

Phosphate buffer 0,01 M / 0,1 M pH 7,2
Dissolve 1,56 / 15,6 g of sodium dihydrogen phosphate dihydrate in 1000 ml water. Adjust to pH 7,2.

Acetate buffer 0,1 M pH 5,2 and 4,0
Dissolve 2 g of sodium acetate in 400 ml water. Dissolve 5,10 g of acetid acid in 400 ml water. Mix the two solutions and dilute to 1000 ml. Adjust pH to 5,2 or 4,0.

Lithium acetate buffer pH 5,2
Add 4,9 g of lithium hydroxide to 20 ml water. Stir vigorously. When half of the salt is dissolved, add 14,65 ml of acetic acid. Stir and let cool to room temperature. Add water to a total volume of 50 ml.
Appendix C

**Standard curve for UV/vis spectroscopy**

BSA

\[ y = 0.0337x - 0.0051 \]

\[ R^2 = 0.9966 \]

<table>
<thead>
<tr>
<th>Concentration (µmol/l)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>1.00</td>
<td>0.05</td>
</tr>
<tr>
<td>2.00</td>
<td>0.1</td>
</tr>
<tr>
<td>3.00</td>
<td>0.15</td>
</tr>
<tr>
<td>4.00</td>
<td>0.2</td>
</tr>
<tr>
<td>5.00</td>
<td>0.25</td>
</tr>
</tbody>
</table>