Preface
The thesis is being delivered to the Chemical Engineering Department at Norwegian University of Science and Technology (NTNU) under Dr. Wilhelm Robert Glomm, Trondheim spring 2014. The project was part of a larger research study of Sulalit Bandyopadhyay, PhD student.

The purpose of this thesis was to study the nanogels as potential drug delivery systems for treatment of diseases in the human body. The loading and release kinetics was studied for experimentally and biologically relevant drugs to/from nanogel networks.

Declaration of compliance
I declare that this is an independent work according to the exam regulations of the Norwegian University of Science and Technology (NTNU).
Place and date: Trondheim, 02.07.2014  Signature: [Signature]
Acknowledgement
I express my sincerely gratitude to my supervisor Dr. Wilhelm Robert Glomm, Senior Researcher at SINTEF and Professor II at NTNU, for his scientific advices and enthusiasm. The understanding of every aspect of the thesis would not have been accomplished without his great ideas and explanations.

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Abstract

Targeting specific drugs to a diseased site is widely studied both in vitro and in vivo, but very few systems have made entry into the clinical market. The systems today cause unwanted side effects due to the lack of specific targeting. This means that a larger dose is required to treat the disease. An interesting option to study within drug delivery systems is the synthesis and proper optimization of Poly(N-Isopropylacrylamide) (PNIPAm), a thermo-responsive polymer. This polymer can be cross-linked with Acrylic Acid (AAc) to form nanogels, which are in the form as hydrogels. PNIPAm/AAc can undergo a volume phase transition at and above its specific volume phase transition temperature (VPTT). This can trigger release of drugs at targeted sites in vivo.

The work described in this thesis focused on studying the loading and release of the PNIPAm/AAc nanogels. The loading has been assumed to occur in the hydrophilic state of the polymer, when the network can contain high ratio of water. In this state the hydroxyl groups of AAc are de–protonated and Coulombic repulsive forces dominate. The drug solution has been introduced to freeze-dried nanogels when they were in the solid state. In this state the polymers can be compared to a sponge which absorbs the solution. This loading mechanism is known as the breathing in mechanism. This mechanism has been used to load two biologically relevant drugs; paracetamol (commonly used experimental drug in the laboratory) and Cytochrome C (a hydrophilic protein which is biologically relevant and whose properties are dependent on pH).

The nanogels have been synthesized, freeze-dried and suspended in solution (1 mg/mL). The properties of these freeze-dried nanogels have been mapped using dynamic light scattering (DLS). The nanogel swelling/de-swelling kinetics have been confirmed to be reversible and the VPTT has been measured at 36 °C (synthesized with 3 mM sodium dodecyl sulphate (SDS) and 8 % N, N’ – Methylenebis(acrylamide) (BIS)) and 39 °C (synthesized with 4 mM SDS and 5 % BIS) respectively.

The loading studies with paracetamol indicated that the drug is relatively hydrophobic. This drug has shown to have higher loading - (61 %) and encapsulation efficiencies (16 mg drug/mg polymer) at elevated temperature, when the nanogel was de-swollen and was in the hydrophobic state. This implied that the nanogel made hydrophobic interactions with the drug. Raising the temperature higher has shown to give squeezing release. The release has also been observed when lowering the temperature below VPTT (when the drug was swollen and hydrophilic). The loading and release studies of paracetamol have also been performed by changing the pH. At pH 3 the hydroxyl groups of AAc is highly protonated (pKₐ = 4.25), which gave polymer/paracetamol interactions and thereby relatively high loading - (60 %) and encapsulation efficiencies (14 mg drug/mg polymer). An increase of the pH to 7 has also given efficient release (46 %) due to the de–protonation of the hydroxyl groups.

In contrast to the measurements of the free (i.e.; not bound) paracetamol for the calculations of loading and release; the bound Cytochrome C was measured after dialysis. Through this method the free Cytochrome C was shown to diffuse through the dialysis membrane, while successful loading and release were proven by measurements of the bound protein. Cytochrome C loading – and encapsulation efficiencies have been calculated to be 86 % and 0.17 mg drug/mg polymer respectively. Release studies of the protein have been performed at 39 °C, and with three
different surrounding pHs: At normal pH conditions, at lowered pH (pH 3) and in PBS solution. The fastest and most efficient release has been observed with lowered pH (24 % release after 24 h).

The nanogels have shown successful loading and release of both hydrophobic and hydrophilic drug molecules by triggering release with change in temperature and pH. This makes them very interesting as drug carriers. The nanogels have the ability to target the desired site with proper modifications, and to exhibit controllable release. This along with stability and degradability of the nanogels can be achieved by modification of the surface. Modification with Poly(Ethylene glycol) (PEG) will avoid early renal clearance of the nanogels. The nanogels can also be incorporated to metal nanoparticles (NPs) which will make it possible to use an electromagnetic field to trigger the release of incorporated drug (in addition to enabled detection and imaging).
Sammendrag
Å rette spesifikke medikamenter til seter ved syke celler er mye studert både in vitro og in vivo, men svært få systemer har gjort inntreden i det kliniske markedet. Systemene i dag forårsaker uønskede bivirkninger på grunn av manglene spesifikk målretting. Dette betyr at en større dose er nødvendig for å behandle sykdommen. En interessant mulighet å forske på innenfor forskning på medikament systemer er syntesen og riktig optimalisering av Poly(N-Isopropylakrylamid) (PNIPAm), et termoresponsive polymer. Dette polymeret kan bli kryssbundet med Akrylsyre (AAc) og danne nanogeler i form av hydrogeler. PNIPAm/AAc kan gjennomgå en volum-fase endring når den gjennomgår overgang ved og over en volumfaseovergangstemperatur (VPTT). Dette kan utløse frigjøring av medikamenter på målrettede områder in vivo.

Dette arbeidet fokuserte på å studere lasting og frigivelse av PNIPAm /AAc nanogeler. Lastingen er antatt å forekomme i den hydrofile tilstand av polymeren, når nettverket kan inneholde høy andel vann. I denne tilstanden er mange av hydroksylgruppene til AAc uprotonerte og de frastøttende Coulombic kreftene dominerer. Medikamentene i løsning har blitt introdusert til nanogeler via frysetørring. I denne tilstand kan nanogeleren sammenlignes med en svamp som absorberer opplosningen. Denne lastemekanismen er kjent som puste-inn mekanismen. Denne mekanismen har vært brukt til å laste to biologiske aktuelle medikamenter; paracetamol (som vanligvis brukes som eksperimentelt medikament på laboratoriet) og Cytokrom C (et hydrofilt protein som er biologisk relevant, og kan avhenge av pH).

Nanogeleren har blitt syntetisert, frysetørrket og re-introdusert til løsning (1 mg/mL). Egenskapene til disse frysetørkede nanogelerne blitt kartlagt ved hjelp av dynamisk lyssprekning (DLS). Den nanogel svellings-/krympings-kinetikken har blitt bekrøftet å være reversibel og VPTT har blitt målt til hhv. 36 °C (syntetisert med 3 mm natriumdecylsulfat (SDS) og 8% N, N'-Metilenbis(akrylamid) (BIS)) og 39 °C (syntetisert med 4 mM SDS og 5% BIS).

Studiene med lasting av paracetamol indikerte at medikamentet er relativt hydrofobt. Dette stoffet har vist seg å ha høyere lastings- (61 %) og innkapslings-effektivitet (16 mg medikament / mg polymer) ved forhøyet temperatur, når nanogelen var i krympet tilstand og i den hydrofobe tilstanden. Dette innebar at nanogelen hadde laget hydrofobe interaksjoner med stoffet. Ved å øke temperaturen ytterligere har en klemme-frigjøring av medikamentet blitt bekrøftet. Det har også blitt observert frigjøring ved senking av temperaturen til under VPTT (i svellet og hydrofil tilstand). Lastings- og frigjøringsstudium av paracetamol har også blitt utført ved å forandre pH. Ved pH 3 er hydroksylgruppene til AAc sterkt protonert (pK_a = 4.25), noe som ga polymer-paracetamol interaksjoner og dermed forholdsvis høy lastings- (60 %) og innkapslings-effektivitet (14 mg medikament/mg polymer). En økning av pH til 7 har også gitt effektiv frigjøring (46 %) på grunn av uprotonerte hydroksylgrupper.

I motsetning til målinger av fri paracetamol for beregningene av lasting og frigjøring ble det bundne Cytokrom C målt etter dialyse. Ved denne fremgangsmåte ble det frie Cytokrom C

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Abbreviations
AAc – Acrylic Acid
BBB - Blood-brain barrier
BIS - N, N’ – Methylenebis(acrylamide)
DLS – Dynamic light scattering
EPR - Enhanced permeability and retention
IgG – Immunoglobulin G
KPS – Potassium persulphate
MFNP – Multifunctional nanoparticle
MWCO - Molecular weight cut-off
NIPAm – N-Isopropylacrylamide
NP – Nanoparticle
PBS - Phosphate buffer saline
PDI – Polydispersity index
PEG – Poly(Ethylene glycol)
PNIPAm - Poly(N-Isopropylacrylamide)
RES -Reticulo-endothelial system
SDS – Sodium dodecyl sulphate
UV-VIS – Ultraviolet visible spectrophotometry
vdw - Van der Waals
VPTT - Volume phase transition temperature
1 Introduction
Most of the drug delivery systems available today for treatment of various diseases are expensive. An exception for this is targeting medicine-capsules (gelatin or matrices). To utilize and optimize these systems, the properties of the drug and its interaction in the body have to be investigated more.[1] Most of the treatment of diseases (including cancer) will not be that specific to the target diseased cells, but spread to the other healthy cells as well. Since a significant amount of the drug will go to these healthy cells, a larger dose is required to have efficient drug to all the unwanted cells. The attack of the healthy cells can also cause side-effects. A good option to avoid this is with targeted drug delivery nanocarriers.[2]

The use of polymeric nanoparticles (NPs) is of potential interest in the field of targeted drug delivery systems owing to multiple degrees of freedom like bio-degradability, hydrophobicity and the form (particles, capsules) in which they can be produced. Targeting specific drugs to a diseased site is widely studied both in vitro and in vivo, but very few systems have made entry into the clinical market. The systems today cause unwanted side effects due to the lack of specific targeting which means that a larger dose is required to treat the disease. A feasible option to investigate in such cases is with nanocarriers capable of targeted delivery of cargo.[2]

These systems allow site-specific drug delivery, in addition to stimuli-responsive control of the release of the drug. They shield the drug and keep it from reacting with the immediate environment. Polymers intended for such application should be able to exhibit low viscosity when injected into the body, but once in the body it should own the mechanical properties characteristic of the polymer matrix. This is possible with polymers sensitive to a change in the external environments.[2]

The polymers have to be incorporated with cell specific markers dependent on the application to reach the desired target. To make the polymer target specific, the pH can be an interesting option to regulate, i.e. in cancer therapy; the drug carriers are easily accumulated in the tumor cells because of their loose junction and insufficient lymphatic drainage.[3] Further, the carriers will be transported into the cell by endocytic vesicles of the cell which change from early to late endocytes. The late endocytes are changed to lysosomes which will have lower pH (~5) than the healthy cells (~7.4). The lysosome trapped drug carriers will then be able to release the drug due to a pH change. This makes it possible to release the drug from a pH-sensitive drug carrier.[3]

In this regard, there has been a tremendous focus on nanogels (polymers in the nanometer range) as a need to understand how thermo and pH responsive hydrophilic polymers can influence potential applications in delivery of specific drugs to targeted locations. Nanogels comprising stimuli sensitive blocks, synthesized in the nano regime yield a wide range of novel properties that can be effectively utilized for various bio-medical applications. The current work has focused on smart nanogels in targeted drug delivery. Multiple stimuli dependence can be achieved by incorporating temperature and pH sensitive blocks in the
polymer backbone. Both pH and temperature dependence can be achieved in a polymer by incorporating the temperature dependent Poly(N-Isopropylacrylamide) (PNIPAm) with the pH-dependent Poly(Acrylic acid) (Poly(AAc)).

The main scope of this thesis is to study the loading and release mechanism of relevant drug molecules from the nanogel network. The lack of knowledge of specific targeting systems is the driving force, since this will cause many side effects and cost barriers. The goal is to deliver a product that can protect the desired drugs from the immune system, target the specific sites and exhibit controllable release.

The loading mechanism chosen is known as the breathing in mechanism which has proven to be an effective way of entrapping the drug in the network. The interaction between polymer-paracetamol is going to be investigated, and hydrophobic characteristics of the drug have been mapped. The interaction between nanogel-Cytochrome C on the other hand showed promising hydrophilic characteristics and was able to release with increased temperature and pH.

These findings make the nanogels potential drug carriers, but the interactions between the network and each desired drug has to be carefully mapped. This can prevent non-specific release of drugs and thereby decrease the toxic effect, in addition to a decrease in the amount of relevant drug. The biodegradable nanogels will also avoid early clearance and hinder the accumulation of drug carrier. This will reduce the costs of the drug carrier systems significantly, which is a large problem with treatment of diseases today.

The characteristics of biodegradation are highlighted since this is a desired quality when introducing an unknown substance to the body. This is followed up by introduction of core/shell nanogel, since these gels are utilized in this study. The loading/release kinetics are also explained, and thereby the characteristics of the interactions between nanogel and loaded cargo. This section comes after the introduction of the two molecules chosen in this study. Thereafter, nanogels with potential for temperature and pH responses with special highlights on volume phase transition temperature (VPTT). A gel network that exhibits a VPTT and used in this study is the PNIPAm/AAc nanogels. The first polymer block is temperature responsive, while the other is pH-dependent. The properties of PNIPAm/AAc polymers are highlighted. These networks can be directly exploited for delivering desired cargo to the target site.

In the first sections the focus will be on microgels (> micrometer) since these larger gels are more studied. However, since the microgels and nanogels have appreciable similarities in physico-chemical properties, the studies with microgels will most likely apply for the smaller gels as well. The consequences of the transition from microgels to nanogels are explained in the last section.
2 Theory

2.1 Nanotechnology in drug delivery

2.1.1 The aim of nanotechnology
In 2010 the global market for drug delivery systems was recorded at 131.6 billion dollars and is estimated to reach 175.6 billion at the end of 2016.[4] The drug’s efficiency and marketability are dependent on the chosen delivery system. New delivery systems have made it possible to give already existing drugs a new chance. The delivery system is usually made with a carrier which often attaches or adsorbs the drug, and then releases the drug upon a change of the external environment.[4] This is shown as an example in Figure 1 by loading cargo to nanogels and release is triggered by a change like temperature or pH.

![Figure 1 – Shows drug loaded nanogels sensitive to external stimuli. Release is triggered by change in the external environment.](image)

Qualities like stability, size distribution and targeting specificity have to be prioritized when designing a drug carrier.[5] Delivery systems within nanotechnology have developed as a huge interest during the last decades. This exhilarating area uses NPs. These particles have the ability to deliver multiple molecules to different sites in the body and keep them sustained over time.[4]

The drug delivery technology has incorporated nanotechnology in many areas. Many NP-mediated therapeutic agents are commercially available. Several NP based therapeutic and diagnostic agents have been developed. Among these systems are treatment of HIV, diabetes, pain, asthma, allergy influenza and cancer. It is especially interesting in the field of cancer research because nanotechnology offers ways of targeting specific sites in the body and to distinguish healthy cells from the diseased.[4] Delivery of down-regulating drugs (drugs that will decrease the receptors on the cell surface) is not desired in healthy cells, and this can be avoided by using NPs as drug carriers loaded with multiple drugs (for instance hydrophobic paclitaxel, DNA, siRNA and hydrophilic doxorubicin) for different functionalities to target different metabolic pathways of the tumor.[6, 7]

When developing these systems, the most important issues are safety and toxicology. Thus, NPs should be appropriately examined for cytotoxicity both in vitro and in vivo. A huge gap between research exploration and nano-scaled pharmaceutical ingredient delivery still exists.[4]
2.1.2 Advantages of NPs
In addition to selective targeting, the specific NPs can reach extended circulation time in the blood, have little immunogenicity, superior biocompatibility, and can possible exhibit competent penetration of barriers such as the blood-brain barrier (BBB).[4] The size, morphology and surface charge of the NPs can also be easily controlled. Another advantage is the possibilities for modifications. For example, the drug can be discharged during delivery and targeting, and the particle degradation can change through proper modifications. Surface modification and size will decide qualities of the NPs, thereby the capacity to release the drug.[4] The incorporation of drug to NPs will give the drug better probability of being transported into the cells via an endocytosis mechanism which is a more efficient transporting mechanism.[8] The endocytosis process of NPs is an activation energy process which involves interaction and collision between the particles and cells. This process is dependent on ionic interactions, and since the cell membranes are negatively charged, the nanocarriers can be modified with positive charge for increased interaction and uptake. The surface charge along with size and hydrophobicity of the particles play an important role in the uptake of incorporated drugs. The size influences the nanocarrier’s intracellular uptake within cells and macrophages.[8]

2.1.3 Administration of the NP
The drug incorporated in the NP has many possibilities and can be administrated through various routes as illustrated in Figure 2. Depending on the content, there are some routes that should be neglected. For instance, oral administration of polyacids will give limited network swelling and slow drug release because of the low pH in the stomach and thereby protonated hydroxyl groups. In contrast, higher pH in the small intestine will cause acid dissociation, so the network will swell and cause drug release in part of the gastrointestinal tract where absorption can occur and where drug hydrolysis is less acute.[9]

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![Figure 2](image-url) *Figure 2 Administration routes of NPs into the body - oral delivery[4, 8], cancer chemotherapy[4, 6, 10], vaccine delivery, ocular delivery, pulmonary drug delivery, dermal and transdermal delivery and delivery systems coupled to implants[4].*
2.1.4 Passive – and active targeting

Once the drug has followed an administration route it will be taken up through either passive or active targeting. Passive targeting exploits the enhanced permeability and retention (EPR)-effect to target tumour cells. This kind of targeting arises because of the circulation of the drug in the blood. The specific drug carriers accumulate near the targeted cells, extravasate, leading to retention in the cell and finally distribute. The increase of the EPR-effect gives better specific targeting, but in the drug delivery this is hard to accomplish. This applies particularly to the tumor cells which change constantly. To increase this effect more information about the distribution of the drug and the dose have to be clear.[11]

Active delivery requires more modification of the surface of drug carriers; bioactive molecules, such as hormones, carbohydrates, peptides and proteins (especially folic acid and its derivatives) have been used as ligands to give nanocarriers specific targeting properties towards drug delivery to diseased cells.[6]

2.1.5 Multifunctional NPs

Surface modifications of the NPs have made it possible to avoid rapid phagocytosis (particularly after intravenous administration). In addition, less drug leakage and thereby less peripheral toxicity can be achieved by surface modified NPs.[4] These surface modifications can provide many properties for the NPs, which are illustrated in Figure 3. These make the NPs multifunctional.[12] In contrast to the conventional drug delivery methods available today, the multifunctional NPs offer drug delivery methods that can co-deliver multiple components, target and possess possibility of simultaneous therapy and diagnosis.[6] A multifunctional NP (MFNP) is shown in Figure 4. The surface functionalities can be varied, and thereby provide different qualities for the NPs. This system will have combined properties, like target specificity, optimized optical, electrical and/or magnetic properties and analysis capabilities.[12]
2.2 Biodegradable drug carriers

2.2.1 Avoidance of the elimination routes in the body

Without the MFNP the drug alone stands small chances once introduced into the body: When a foreign object is introduced to the body, the filtration in the kidney and the premature clearance through the reticulo-endothelial system (RES) are the most significant challenges.[5] These elimination processes are shown in Figure 5. The kidneys will eliminate particles smaller than 8 nm, and in addition the spleen and liver will capture particles larger than ~200 nm.[13] The renal clearance pathway is an efficient elimination method. The particle molar mass is of great importance, but in addition, the dimension, hydrophobicity and surface charge also have to be taken into account.[13] Unknown substances (for the body) have to go through several barriers before it reaches the molecular site of action. How the drug is transported in the vasculature and tissue is decided by convection in the circulation and diffusion and convection in the tissue interstitium (between the blood - and lymphatic capillaries). If it escapes from the barriers created in the blood circulation, and reaches the target (the probability for accumulation of drug is higher in tumor cells because they have leakier vasculature compared to healthy cells), it has to survive the harsh acidic environment of endolysosomes and go through the nuclear membrane. In these conditions it is most likely that proteins, oligonucleotides and other bio-molecular drugs will be inactivated or degraded. Pathological cells can also develop multiple drug resistance.[5]
2.2.2 Degradation
To avoid the drastic removal of the polymer by the body’s defense mechanism, biodegradable polymers are desired. These polymers are complicated and they should not cause unwanted responses in the body. The degradation should also happen in a controlled manner.[1] The decrease in the size can cause variations in the physiochemical and structural properties. This can lead to numerous material interactions which may produce toxic effects.[4] The decrease in size will make the degraded particle have different properties when it is decomposed. The decomposition can be tuned by modifying cross-linker density within the network.[13]

2.2.3 Triggering release by degradation
Degradation of drug carrier can be used to trigger release of drugs. Self-degrading drug carriers can be tuned to have a release rate that can go anywhere between seconds to days. This is possible by varying the composition of the drug carrier. Related to the self-degradation is the chemical and/or enzymatic triggering, which is interesting in cancer therapy. Change in pH can break disulfide cross-links, and the polymer will then degrade. The cross-linking plays an important role when degrading a polymer[9], as the cross-linker has proven to influence density, size and mechanical properties within a polymer network.[16]

2.3 Core/shell hydrogels

2.3.1 pH – and temperature–responsive polymers
Chemical degradation has been partly achieved with biodegradable pH–responsive Poly(AAc) microgels. These microgels were cross-linked with disulfide groups that swelled by chemical reduction of the disulfide bonds. The chemical degradation was much similar to the physically cross-linked gels, but this proves that the microgels can be triggered to swell by rupturing cross-links either by physical or chemical parameters.[9]

The microgels offer drug delivery systems that have advantages such as conformational stabilization of the drug. They introduce efficient loading and release of drugs and in the years
to come, the microgels will be more focused on as delivery systems for bio-macromolecules (such as proteins, peptides, siRNA etc.).[5, 9] This is due to the discovery of their ability to provide stability through secondary and tertiary structures, avoidance of aggregation and chemical/ enzymatic degradation. In addition they can reduce toxicity and other biological effects, as well as retain biological activity of the drug. The understanding of how to utilize these qualities of the microgels for loading and release has been focused on in the last years. The microgels can be functionalized in order to be triggered by multiple and diverse factors. The gel’s interactions can be controlled by pH, hydrophobicity and charge. Tuning these factors correctly, a homogeneous and predictable microgel with the desired properties can be achieved.[9]

2.3.2 Conformational changes of PNIPAm/AAc

The microgels of Poly(AAc) are pH-dependent. Above the pK_a-value the AAc–polymer interactions will be enhanced and impaired below pH 3. At the latter pH, it has been observed that anionic microgel films in multilayers did not load any molecules.[16] These microgels have been cross-linked with PNIPAm and biodegradable disulfide groups. These microgels showed to swell up by chemical reduction of the bonds. In addition, biodegradable cross-linked PNIPAm/AAc have shown degradation–limited swelling kinetics.[9] The PNIPAm is a widely studied temperature-responsive hydrogel.[13] PNIPAm exhibits low cytotoxicity.[17] The polymer has increased surface charge density over the transition temperature, and the major driving force for collapse is hydrophobic interactions.[8] PNIPAm alone is a non-biodegradable polymer.[17] This quality of PNIPAm is due to its tendency to self-crosslink during the synthesis. This makes PNIPAm-network unpredictable and non-degradable which is not a quality desired in drug delivery.[18] There exist uncertainties regarding the toxicity of PNIPAm which have to be further investigated.[9] Another disadvantage of this polymer is that smart drug delivery systems based on this polymer show slow swelling/de-swelling transitions. This is due to formation of hydrophobic skin that inhibits the release of drug and limits de-swelling of gels. A method to avoid these disadvantages is to incorporate hydrophilic polymer into the hydrogel, which will enhance the flux of water from the bulk which will therefore permit more efficient collapse. Hydrophilic polymers that are acidic or basic have been incorporated to the polymer chain, which make them dependent on pH. Depending on the pK_a-value, the solubility of the polymers will change. These synthetically, especially acid-based, polymers are mostly developed with relation to drug delivery.[17] The incorporation of a hydrophilic co-monomer can also make the polymer network degradable.[18] Comparing PNIPAm to PNIPAm/AAc, the latter has larger free volume and diffusion may be easier through the polymer shells and the carboxyl groups of AAc introduce tunability of its properties.[19]

When the lightly cross-linked PNIPAm/AAc particles are exposed to change in temperature or pH they have the ability to undergo drastic changes in the conformation. This is illustrated in Figure 6 and this gives qualities that enable the gels to deliver bio-macromolecular drugs in a controlled manner.[9]
Temperature- and pH-dependent polymers are highly popular in drug delivery research. Different pH environments will create different interactions.[16] Close to the pHₐₐₜ value of AAc monomer (pKₐ = 4.25), an increase of the AAc ratio will give a decrease in the VPTT of PNIPAm/AAc. In addition, close to pKₐ, the average particle size increases significantly, while the zeta potential is lowered. This is due to the fact that at higher pH values, a pH-responsive swelling behavior is observed owing to the de-protonation of AAc segments which leads to electrostatic repulsion between carboxylate anions. This causes an increase in the osmotic pressure inside the particles, thereby increasing the swelling of the polymeric networks.[21]

![Figure 6](image_url)  
*Figure 6 – Shows the size as a function of a) temperature and b) pH.[20]*

2.3.3 Interactions between core and shell

The temperature – and pH responsive polymers can be modified to have multiple orthogonal functionalities, which the core/shell structure gives a robust platform for.[16] Core/shell particles introduce controlled (shell-mediated) biological interactions in addition to good carrier (core-based) properties. The core and shell are mechanically bonded together and influence each other physico-chemically. This is especially true for the polymer PNIPAm, which is thermo-responsive due to thermodynamic coupling. The core and shell desolvation will influence each other.[22] Research with PNIPAm has shown radial distribution of connectivity with higher density of polymer in the core.[13]

In a study, both core and shell were synthesized in aqueous solution at 70 °C. The collapsed PNIPAm microgel core served as nuclei for the growth of the PNIPAm/AAc shell. Both transmission electron microscopy and dynamic light scattering (DLS) were used to observe the properties of the gel. The gel showed low polydispersity and it was confirmed that
addition of the shell showed a particle size increase. The gel also showed a sharp interface without significant interpenetration between the core and shell. In addition, studies have shown that PNIPAm/AAc particles have energy transfer across the core/shell interface when they collapse. These core/shell nanogels have been optimized through the synthesis towards drug delivery by adding alkyne or azide groups which can give the gel the appropriate functionalities for the desired applications.[16]

2.3.4 Influences of core and shell
Multi-responsive PNIPAm/AAc gels have been synthesized proving that the distribution of the functional groups is important. The introduction of PNIPAm/AAc as core particles has proven to increase the original VPTT-values when increasing pH from 3.5 to 6.5 because increasing the pH causes de-protonation of AAc. The increased solvated ions will increase the swelling and inhibit chain collapse of the particle. This result will again lead to increased osmotic pressure and Coulombic repulsion. In addition to the PNIPAm/AAc core particle, a PNIPAm shell has been introduced. The shell collapsed at 32°C, and hindered the core collapse. This hindrance was increased at pH 6.5 because of the shell-dominance. The shell has a compression effect which leads to decrease in the average inter-chain distance in the core and thus decreases the VPTT of it. This study was also performed with PNIPAm in the core and PNIPAm/AAc in the shell. The shell was not much influenced by the core in this study. The end conclusion of these two studies was that when looking at the characteristics of these core/shell gels, it is just as important to look at the whole exterior as each individual component.[16]

2.3.5 Location of incorporated drug
Recent studies of core/shell particles in the form of gels have shown more binding events on the periphery of the particles in the gel compared to the interior. This can be explained by the more reachable and available sites on the surface. The interior of the particle is restricted by the polymer network and is therefore less reachable for the drugs.[16]

2.4 Paracetamol
One drug commonly used in laboratory experiments as a standard drug is paracetamol. This drug is also called acetaminophen, and has the systemic IUPAC-name N-(4-hydroxyphenyl)acetamide.[23] The structure is shown in Figure 7.

![Figure 7 – The chemical structure of Paracetamol][24]

This drug is used as a pain reliever (analgesic) and fever reducer (antipyretic). Post-surgical pain and providing palliative care in advanced cancer therapy can also be managed by this drug. It is in the class of “aniline analgesics” and does not exhibit significant anti-inflammatory activity. In therapeutic doses, it is not considered carcinogenic. The way
paracetamol operates is not yet completely known. The known factor is that it works as an inhibitor of cyclooxygenase, and that it is restricted by for instance high level of peroxides present in inflammatory lesions. To convert it into a non-toxic drug, paracetamol follows three metabolic pathways as can be seen in Figure 8.[23, 25] These pathways consist of Glucuronidation (2/3 of the entire metabolism), Sulphation (sulphate conjugation) and N-hydroxylation followed by Glutathione conjugation.[23]

Figure 8 – The metabolic pathways for paracetamol.[25]

These pathways give products that are non-toxic and inactive, and they will be excreted by the kidneys. However, in the third pathway a toxic intermediate product; N-acetyl-p-benzo-quinone imine which is an alkylating metabolite is produced. It is further irreversibly conjugated by Glutathione’s sulphydryl groups, but in the intermediate state it is a form of toxification.[23, 26]

Paracetamol can be recognized using the Ultraviolet visible spectrophotometry (UV-VIS) and the major absorbance peak of paracetamol is observed at a wavelength of 243 nm.[23] The solubility of the drug is expressed in Equation 2.1.

\[ C_s = \frac{10^3 (m_{vdr} - m_v)}{m_{vs} - m_{vdr}} \]  

(2.1)

Where \( m_{vdr} \) is the “dry residue” mass, \( m_v \) the sample vial and \( m_{vs} \) the sample vial with the saturated solution. The solubility of the drug has been determined by Roger A. Granberg and Åke C. Rasmuson, which is shown in Table 1. This solubility applies for the drug in water.[27]

Table 1 – Shows the solubility of paracetamol (\( C_s \)) given as g paracetamol/kg of water at the corresponding temperatures.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>0°C</th>
<th>5°C</th>
<th>10°C</th>
<th>15°C</th>
<th>20°C</th>
<th>25°C</th>
<th>30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_s )</td>
<td>7.21</td>
<td>8.21</td>
<td>9.44</td>
<td>10.97</td>
<td>12.78</td>
<td>14.90</td>
<td>17.39</td>
</tr>
</tbody>
</table>
Although paracetamol has an appreciable solubility in water, it is hydrophobic than many other clinically relevant drugs.

2.5 Cytochrome C
Another drug commonly used and which is more hydrophilic than Paracetamol is Cytochrome C. This molecule is illustrated in Figure 9.

![Figure 9 – Illustration of Cytochrome C][28]

Cytochrome C is a hydrophilic protein important for cellular oxidation and is a universal link in the respiratory chain as it forms electron-bridge between respirable substrates and oxygen. The mitochondrial drug is released into the cytoplasm and stimulates apoptosis, and is considered an important mediator in apoptotic pathways.[29] Cytochrome C is a biologically relevant molecule showing wide conformational variations at different pH conditions and is quite ideally suited to study interactions at clinically relevant pHs. The isoelectric point of Cytochrome C is at pH 10 – 10.5.[30] At neutral pH the protein exhibits positive charge (+8). This molecule is a basic redox-heme-protein and plays an important role in the biological respiratory chain.[31] Cytochrome C is an efficient biological electron carrier due to ready interconversion of it between ferrous and ferric states.[29] This protein’s heme-group shows a characteristic UV-VIS peak at 409 nm.

2.6 Loading of the drugs

2.6.1 Definition of loading and release
To load the drugs, freeze-dried polymers can be used. Loading doses is often higher than the dose delivered and is administrated to establish therapeutic level of medication.[32] Successful loading can be achieved by compatible carriers and appropriate location of the molecules in/on the carrier. The stability of the molecules is important during loading, storage and release of them. This will especially apply for proteins, peptides and oligonucleotides since they may lose their biological activity.[33] Sustained release systems give prolonged time of drug molecules in the blood or tissue. Sustained controlled release is necessary to give desired drug concentration to the target tissue or cells. Controlled release is defined as rate-controlled drug delivery. These systems have an ability to specify the release rate and duration in vivo.[34]
Loading and release have proven to be influenced by different bindings between the drug and drug carrier, and in the form of a gel network these factors will be influenced by size, cross-linking density and network homogeneity.[9]

2.6.2 Loading – and encapsulation efficiency
The loading of drug and the loading mechanism onto PNIPAm/AAc particles is dependent on the ratio of cross-linker and AAc.[9] In addition, the heterogeneity and hydrophobicity of the polymer network will influence the loading/release kinetics. For example in a study by Bysell et al. the swelling kinetics decreased when increasing the hydrophobicity with hydrophobic modification of PNIPAm microgel.[9] This gave both smaller temperature-induced collapse and lower rate of de-swelling. By adding voids to the PNIPAm microgels the temperature response increased, which indicated that the (de)swelling kinetics can be tuned by the size and number of voids. This influence of heterogeneous gels makes it important to make them monodisperse and uniform.[9] In addition, the loading is dependent on the type of surface-active materials and stabilizers present.[4]

The amount loaded is usually presented in mg drugs/mg polymer. The loading efficiency is important, and the amount of carrier administrated should be minimized.[4] The calculation of loading efficiency and encapsulation efficiency is shown in Equation 2.2 and 2.3 respectively.

\[
\text{Loading efficiency: } \frac{\text{Amount drug loaded}}{\text{Amount total drug added}} \cdot 100\% \tag{2.2}
\]

\[
\text{Encapsulation efficiency: } \frac{\text{Amount drug loaded}}{\text{Amount polymer added}} \tag{2.3}
\]

2.6.3 Loading methods
The loading methods can be divided into these categories[4]:

1. Incorporation method, where the drug is integrated at the time of polymerization.
2. Adsorption method. The drug is incubated in solution and introduced to the polymers in solid form. The amount adsorbed is dependent on the drug–polymer affinity.
3. The breathing in mechanism, where the polymers are loaded by putting the polymer powder in a drug-concentrated solution. The solution volume is adjusted so the gel swells up the whole volume.[13] This method is the more popular one in case of nanogels and would thus be discussed in detail.

The breathing in mechanism has been used to load the macromolecular therapeutic agent insulin by Nolan C.M. et al. It was proven that the breathing in mechanism gives a more dramatic swelling response used for loading, and entraps solutes in hydrogel networks. This will allow the solute molecules to partition into the porous network. The breathing in
2.6.4 Interactions between the carrier and the drug

The loading (as well as release) of drug from a polymer network is very much dependent on the interactions between them. This was confirmed by a study with PNIPAm functionalized with amine to give surface charge, and without affecting the temperature dependence. The human Immunoglobulin G (IgG) was then adsorbed to the polymer; at low temperatures (swollen PNIPAm) the adsorption was low as a consequence of low Van der Waals (vdw) interactions between polymer and drug. Above the transition temperature, PNIPAm is denser, which leads to higher vdw–interactions and thus higher adsorption of IgG.[9]

Hydrophobic – and non–electrostatic interactions are of great importance when loading amphiphilic drugs.[9] However, the most important interaction is the ionic interaction between the drug and the carrier as the drug enters the binding seat.[4] Many studies have proven that introducing an ionic interface between drug and matrix will give efficient drug loading. The ionic interface will be inversely related to the distance between two charged atoms, and the environment also has to be taken into consideration. Hydrophilic interfaces and equilibrium of electrostatic interaction have shown to give proper adsorption and release.[4]

The microgel loading of hydrophilic and charged bio-macromolecules will be easier if the hydrogel is hydrophilic, or has some hydrophilic compartments. This kind of gels also provides a hydrophilic matrix. This will not cause significant conformational changes and aggregation of proteins and peptides, although this depend on interaction strength, in contrast to hydrophobic surfaces. This advantage of (partly) hydrophilic hydrogels helps bio-macromolecules maintain their biological effect. This effect has also been proven when incorporating an enzyme to the hydrogel; the enzyme alone loses biological activity, but no loss in colloidal stability is observed when incorporating it to PNIPAm microgels. In addition, less conformational changes are observed when increasing the temperature and higher thermally stability for the microgel-loaded enzyme.[9]

The nature of the microgel is not the only factor controlling the drug loading efficiency but also the drug itself. It has for example been shown that polyelectrolytes with lower pK_a-value adsorb better than those with higher pK_a–value. This is probably due to the fact that weak polyelectrolytes are more coiled up at intermediate pH. In contrast, the strong polyelectrolytes exhibit strong interactions and thereby topological restrictions and the result of fewer electrostatic bonds and larger average pore size, thereby less efficient loading. Inefficient loading will also occur if the drug forms a shell on the microgel.[9]

2.6.5 Loading with peptides and proteins

The shell formation around the microgel has been confirmed through a study with peptides incorporated to Poly(AAc) microgels . This study was performed to clarify the drug’s ability
to influence the loading. Here, it was shown that the size of the peptides played an important role for loading; small peptides seemed to distribute evenly throughout the gel, while larger peptides formed a shell around the microgel. In addition, at pH 4.5, when the hydroxyl groups are protonated, smaller peptides were needed to form a shell. This is due to lower degree of dissociation and correspondingly smaller mesh size before peptide binding. The conclusion from this study by Bysell et al. was that the peptide–microgel binding depends on the peptide size and the degree of charged hydroxyl groups. This is the general rule, but shell formation is also dependent on peptide charge densities and distribution, pH and ionic strength. The degree of peptide incorporation affects loading, release rate and chemical and enzymatic degradation of the peptide. It has also been proven that the peptides can be cyclized without affecting the loading and release, and can improve proteolytical and chemical stability or other advantages of cyclic peptides compared to linearity.[9]

The polyelectrolytic peptides have a relatively uniform charge density, they should avoid shell formation and the release of them should be triggered and controlled. There exist many research papers with microgels and peptide/protein as drugs.[9] Proteins and peptides are hydrophilic and charged, which means that electrostatic interactions are important between them and charged gels. A study with bovine serum albumine on PNIPAm/AAc showed that maximum adsorption on the gel was close to the isoelectric point of BSA. At this pH, the lowest inter-protein repulsion is present. In addition, when the gel is more charged, the adsorption of the polypeptide increases, but at a certain point of charge it starts to decrease.[9]

2.6.6 Charge localization
The efficiency of both loading and are also dependent on the localization of charge on the drug: Charge localized on one part of the drug has proven to give more significant deswelling of the polymer compared to drugs with even distribution of charge. The drug with charge at one part has also shown better loading, but at high ionic strength the release was better for the evenly distributed drug. This study was performed with fully charged Poly(AAc/Acrylamide). To compare, low-charged microgels (25 %) did not have the ability to differ these differently charged drugs.[9]

2.7 Release of the drugs
2.7.1 Release mechanisms
The principle of a stimuli responsive release of the drug is illustrated in Figure 10. The release can either happen by “squashing release” of the incorporated drug or reduced drug release due to entrapment. This possibility of variation can give triggered drug release. In addition, a temperature-dependent gel, like PNIPAm, can vary the activity of the drug when it goes from swollen to de-swollen state.[9]
For efficient release, it is important that the drug is homogeneously spread out in the matrix of the carrier. The release rate depends on desorption of the surface bound drug and diffusion of it from the carrier. Drug release occurs mainly by diffusion if the spreading of drug is more rapid than degradation of the carrier. If it is opposite, the release is dependent on degradation.[4]

2.7.2 Triggered release
Controllable release of the drug can be achieved, by for instance utilizing an electrostatic trigger to release the drug. The electrostatic interaction plays a huge role in the loading capacity of the gel and contributes for possibility of triggered release. If an electrostatic trigger is used, the charge contrast between the drug and gel has to be moderate at physiological ionic strength to avoid incomplete drug release. Moderate de-swelling transitions (through control of charge density, cross-linking density etc.) are also required. A related study to this used an electrolyte which caused dissociation of the hydroxyl groups of the polymer which again led to expanding of the microgels. This type of expansion has proven to give better drug release. Another study showed that when utilizing the electrostatic triggering correctly, the drug release can be triggered by pH. This has been confirmed by Bysell et al. with PNIPAm/AAc microgels modified with transferrin-based cancer targeting specific to HeLa cells.[7]

2.8 Hydrogels and VPTT
2.8.1 Characteristics of the hydrogels
PNIPAm/AAc hydrogels have proven to have the ability to be precisely controlled by small changes in temperature. This is one of the reasons why these hydrogels are good model systems to study cellular uptake.[8] Drugs can be loaded and released from hydrogels that exhibit a VPTT. This is one of the best achievable properties of the hydrogel’s constructs.[13]
When the gel network goes through a de-swelling it can reorganize, causing dramatic changes in the surface chemistry or energy. This is a good quality in cellular therapy.\cite{13} The gel has the ability to swell and de-swell almost reversibly, causing changes in the surface chemistry and energy, due to expulsion of encapsulated solutes.\cite{16} In a solution the de-swollen gels still consist of water, typically ~20\%.\cite{18} These hydrogels are excellent drug carriers as they exhibit similar Young’s modulus to that of the cell’s extracellular matrix. The hydrogels have tissue-like mechanical properties, and the ability to contain large fractions of water that make them capable to resist protein adsorption or cell adhesion.\cite{16} Nano-sized hydrogels, nanogels, have proven to avoid many side-effects (like toxicity and non-specific targeting) that come with the treatment for diseases today.\cite{37} Hydrogels that respond to external stimuli are important due to the ability for controlled release of drugs.\cite{17}

2.8.2 Temperature dependent transition
The temperature of the conformational change of the hydrogel is referred to as the VPTT. The VPTT describes a network that goes from a highly swollen state to a collapsed and dehydrated state. This phenomenon is driven by entropy. This is a good advantage in drug delivery, as it can control uptake and release of drugs. That is why polymer networks that exhibit VPTT is one of the most studied drug delivery systems.\cite{35} The volume phase transition can be triggered by temperature, as seen in Figure 11. This figure shows a reversible swelling transition. Over the VPTT the polymer will be in the collapsed state, expelling the water. Below the VPTT it will be hydrated and swollen.\cite{13, 38}

![Figure 11 - Temperature dependent swelling and de-swelling of a hydrogel network\cite{38}.](image)
2.9 Transition from microgels to nanogels

2.9.1 Advantages of the nanogels

In drug delivery research there is a profound literature on microgels.[9, 22, 35, 39] The microgels have shown to reduce chronic inflammation, they have been successfully used in sensing, drug delivery and as implantable biomaterials. These microgels have a larger size range than the nanogels. Both gels can be produced by almost similar synthesis methods, but with different reaction conditions and mole ratios of the reactants. The microgels and nanogels have similar physical and chemical properties, but the smaller size will give nanogels the ability for better controlled release.[40]

There is a limited number of studies of nanogels on the other hand[16], although they offer many advantages over these larger gels: In contrast to the large hydrophilic molecules, they can penetrate membranes to access the cytoplasm, and they are not filtered by the kidneys as fast as the larger ones. [5] In addition, bioaccumulation can be reduced and the clearance by renal filtration can be improved.[37] Small gel networks can also be designed to respond to different stimuli, [9] and it is easier to create complex interfaces with smaller gels.[16] In addition, the tiny size and mobility of NPs make them able to locate wide ranges of targets.[4]

The nanogels are always in the form of hydrogels. Advantage of small gels (~50-200 nm) is that they are favored in case of intravenous drug delivery.[16] They can also cause fast response (while injected directly into the blood for example), which also opens for opportunities in other delivery routes.[9] Intravenous delivery has been done with nanogels consisting of PNIPMAm. These gels have shown to encapsulate, retain and deliver siRNA to cancer cells, while still being relatively non-toxic.[37] When the drug carrier is injected into the body, particles will avoid being captured in the tissue interstitium, and rather be taken up by the interstitial flow (fluid flow between blood capillaries and draining lymphatics), see Figure 12.[5]

![Figure 12 – Shows the location of the interstitial fluid.[41]](image-url)
2.9.2 Elimination of NPs
The clearance by renal filtration is the most effective NP elimination route in the body. Other elimination methods of the NPs involve liver sequestration and subsequent reticuloendothelial macrophage uptake and hepatobiliary excretion into the intestine. Besides elimination by clearance, erosion is a possibility which can also modulate the release of drugs through network decomposition. This most likely changes the colloidal properties of the gel, and it is essential that it is monitored during degradation of the polymer.[37]

Studies with nanogels have shown that the gels can erode depending on both pH and temperature. The nanogels showed higher degradation at higher pH and at elevated temperatures. This tunability of the drug carriers makes them ideal towards intravenous drug delivery. The particles also showed loss in light scattering, which means that the particles undergo loss of mass as well as overall decrease in particle number density.[37]

2.9.3 NPs as nanogels
In the recent years, NPs in the form of nanogels have been under high focus for targeted drug delivery applications.[16, 37] The conventional drugs used today in drug delivery have poor water solubility, rapid clearance from circulation, low bioavailability, and inefficient cell entry.[37] The porous polymeric nanogels consist of cross-linked polymer chains which are formed by either self-assembly or covalent linkages. Due to the porosity of the gel, it can contain high amounts of drug, compared to other organic carriers such as micelles or liposomes. It has also the advantage of changing its morphology when introduced to a change in the external environment. These qualities are highly desired in drug delivery research as it improves the in vivo delivery.[6]

In general the NPs have shown higher intracellular uptake compared to the micro-sized particles. Several in vitro studies have indicated that the particle size can control cellular uptake. For instance, NPs larger than 230 nm have shown to congregate in the organ (especially spleen) due to the capillary size of the organ. Small changes of the size of the NPs can influence their cellular uptake and bioavailability.[4]

2.9.4 Nanospheres and -capsules
Comparing gels as nanospheres with -capsules one can describe the spheres as less complicated. The sphere and capsule is shown in Figure 13 In addition the spheres have more of an abrupt volume transition due to connectivity throughout the particles. Physiochemical interactions and covalent bonds in the gels allow stimuli-controlled drug release.[9] This release, in addition to the uptake, can also be tuned by the porosity of the polymeric network (mesh size). The porosity can be controlled by cross-linking density and electrostatic repulsion (increase/decrease).[16]
2.9.5 Nanogels with surface functionalities

The advantage of nanogels is their small size compared to the conventional delivery systems today, which contributes with *in vivo* solubility and stability for the drugs injected and more drugs being able to penetrate the cellular membrane of the target. Due to the nanogels’ small size, the surface to volume ratio is high which means that the huge surface area can provide many opportunities when functionalized.[6] This high surface area means that the majority of the drug will be coupled with in or near the exterior of the nanogel which will result in fast release.[4]

This high surface area characteristic of the nanogels can make them specific to targeting. The surface can be modified to own properties that will give the nanogel longer circulation time in the blood with induced targeting delivery. Other surface functionalization can give the nanogel capability of penetrating the tumor cell, gain diagnostic properties and/or help target the desired cell.[6]

2.9.6 Uptake of the nanogels

By adding desired properties, active targeting can also be promoted for nanogels.[16] Studies have implied that nanogel uptake is primarily driven by Ephrin type A receptor 2 binding (a gene).[13, 43] The chemosensitization has been proven to be increased with just the nanogel, but the total therapeutic effect was influenced by this and the loaded drug (in this case siRNA).[13]

Nanogels offer high swelling qualities, non-fouling characteristics and have shown to be able to retain and release macromolecules. They are capable of intravenous administration as colloidal dispersions and they show similar properties to that of the bulk synthetic hydrogels.[37] Studies have shown that it is possible with faster de-swelling kinetics than macrogels, making the reaction to external stimuli more rapid with smaller gels.[16]
3 Materials and methods

3.1 Material

3.1.1 Reagents

Sodium dodecyl sulphate (SDS), Potassium persulphate (KPS), N, N’ – Methylenebis(acrylamide) (BIS), N-isopropylacrylamide (NIPAm), Acrylic Acid (AAc) (1.051 g/mL), Cytochrome C from bovine heart, Monopotassium phosphate, KH₂PO₄ (50 mM) and Phosphate buffer saline (PBS) have been purchased from Sigma Aldrich. Paracetamol has been purchased from Weifa and Di-Potassium hydrogen phosphate trihydrate (K₂HPO₄) (50mM) and n-Hexaan from Merck Millipore.

3.2 Characterization methods

3.2.1 DLS

3.2.1.1 The principle of DLS

Light scattering can be used to analyze the structure of the hydrogel (phase-transition behavior, mass transport through the network, colloidal stability etc.).[44] The DLS measures the intensity of the scattered light, which is a fluctuating quantity as a result of Brownian motions of the suspected particles.[45]

The DLS can be used to measure the size, distribution and diffusion coefficient of a polymer solution. This can also be called a photon correlation spectroscopy, and is a time-dependent light scattering.[46, 47] It is a quick method that characterizes the hydrodynamic size and analyzes the response and stability of the particles.[46] The intensity of the center of the scattering varies because of the random motion of the particles.[47] The diffusion coefficient in a dilute dispersion (measures the interactions between particle and solution) is described in Equation 3.1.

\[
D = \frac{k_B T}{6\pi\eta R_H}
\]  
(3.1)

Where \( k_B \) is the Boltzmann constant, \( T \) is the absolute temperature of the diffusion, \( \eta \) is the intrinsic viscosity and \( R_H \) is the hydrodynamic radius.[47]

3.2.1.2 Nano Sizer

An instrument used to measure DLS is the Nano Sizer. This is a particle size analyzer which can measure the molecular weight or size of the particles. It has a range from below a nanometer up to microns.[48, 49] The Zeta Sizer in the Nano Range (Nano Sizer) is shown in Figure 14 a, and uses DLS to measure the size. The principle of the measurement of this instrument is illustrated in Figure 14 b.[50] The size is calculated from the diffusion coefficient of the particles that move by Brownian motions by Stoke-Einstein's relationship.[51] The instrument also measures the zeta potential, \( \xi \), by following the Smoluchowski equation given in Equation 3.2.[52]

\[
\nu_E = 4\pi\varepsilon_0\varepsilon_r \frac{\xi}{6\pi\mu} (1+\kappa r)
\]  
(3.2)

Where \( \nu_E \) is the mobility of the particles in an electric field, \( \kappa \) is the Debye-Hückel parameter,
$\varepsilon_0$ and $\varepsilon_r$ are the relative dielectric constant and the electrical permittivity of vacuum respectively, $\mu$ is the solution viscosity and $r$ is the particle radius.[52]

![Diagram](image_url)

**Figure 14 – a) Zeta Sizer, Nano Range ZS[50] b) Measurement of a sample in the Zeta Sizer.[53]**

To determine the zeta potential, laser doppler micro-electrophoresis is used. This sets up an electric field in the solution which triggers the molecules to move. The electrophoretic mobility is calculated from this using a phase analysis light scattering, and from this the zeta potential is calculated.[50]

### 3.2.1.3 Nano Sizer measurements

The Nano Sizer was turned on with the setting to measure both the size of the particles and their zeta potential. The zeta potential was measured in the zeta cuvette and the size was measured through the glass size cuvette. The synthesized solutions were diluted (100 times) with filtrated deionized water before measuring.

### 3.2.2 UV-VIS

#### 3.2.2.1 The principle of Ultraviolet–Visible Spectrophotometry

The amount of drug loaded to the polymers can be calculated based on the results from the Ultraviolet-Visible Spectrophotometry (UV-VIS). The UV-VIS measures the amount of ultraviolet or visible radiation absorbed by a substance in solution. This instrument can give both quantitative information with use of a calibration curve and a qualitative analysis by calculations of the absorbed radiation.[23, 40] The UV-VIS gives possibility for rapid analysis of small concentrations based on the Beer-Lambert’s law. This law is expressed by Equation 3.3.[23]

\[ A = a b c \]  
(3.3)

Where the absorbance/optical density is $A$, absorptivity/extinction coefficient is $a$, the path length of radiation through sample (cm) is $b$ and $c$ is the concentration of solute in solution). The only variable is the concentration.[23]

The principle of UV-VIS is to measure the absorbance of a solute in a transparent solution at a suitable wavelength. This is dependent on the nature of the sample and is normally chosen around the substance’s maximum absorption. The absorption should be adjusted to ~0.9 to optimize the accuracy and precision of the measurement.[23] The absorbing component can
be calculated by using one of the three procedures; standard absorptivity value, single or double point standardization and calibration graph. The first one is used when it is difficult to get a sample of a reference substance. The second is a measurement with a standard solution and a solution with the reference/standard substance. The last one is usually done before the second procedure. It involves standard solutions with known concentrations and the corresponding measurement of the absorbance.[23] In recent studies, a main problem with this kind of sensing systems has been false positive results due to specific secondary binding or non-specific adsorption of other species in solutions.[16]

3.2.2.2 Analysis
The UV-VIS was switched on and a baseline was made before measuring the solutions. The cuvettes used were made of quartz and they are shown in Figure 15.

3.3 Methods
3.3.1 Recrystallization of NIPAm
The NIPAm was recrystallized using a setup shown in Figure 16. The one- necked glass flask was cleaned with n-hexane, before adding 50 mL of n-hexane (for 5 g of the monomer) to the flask. Recrystallization was done at 110°C for 2 hours.

The reaction vessel was thereafter put directly in an ice bath for 20 minutes. The solution was then filtered using a Filter Paper Circles (90 mm). When the monomer was dry the sample was weighed and stored in the refrigerator for further use.
3.3.2 Precipitation polymerization of the PNIPAm/AAc nanogels

3.3.2.1 Principle of synthesizing PNIPAm/AAc nanogels

Synthesis of PNIPAm/AAc was started with the initiator KPS. The initiator helps stabilize the polymers to the critical size, a point where the initiator does not have any more charge to stabilize more polymers. The initiators of the KPS are the sulphate radicals, which are activated at a high temperature (~70 °C). The radicals attack the monomers which start a radical propagation and chain growth. The growing chains reach a critical length, collapse and form precursor particles. These particles are captured by other particles and growth by aggregation can occur. The particle size can be decreased by adding an ionic surfactant like SDS, which stabilizes the particles earlier in the reaction.[44] The principle of the free radical precipitation polymerization is illustrated in Figure 17.
The concentration of the monomer and stabilizer, as well as the stirring speed are important factors of how the NP are formed.[55]

3.3.2.2 Procedure of synthesizing PNIPAm/AAc
The procedure used in this project is adapted and modified from the one reported by Tam et al.[56] The concentrations of the components are modified from the procedure above to give smaller particle size. When synthesizing the PNIPAm/AAc nanogels used in this study the mole ratios between PNIPAm, AAc and BIS need to be known. The molar composition used was 85 % PNIPAm, 10 % AAc and 5 % BIS, which concurs with the composition used by Lyon and Singh to synthesize nanogels.[19] The AAc was stored in solution (1.051 g/mL), so the amount in mL was calculated from the diluted AAc solution (0.1051 g/mL). The basic modifications and calculations have been done in previous work at the Ugelstad Laboratory.[57]

3.3.2.3 Synthesizing PNIPAm/AAc
The new and modified procedure is as follows: A one necked glass-flask (25 mL) was equipped with a nitrogen inlet which was then put to low degassing during the entire reaction. The reactor was de-oxygenated with nitrogen before and after the solution was added, and left on during the reaction. This was to avoid formation of unwanted products. To avoid this, it was also important to use clean water in the solutions. The deionized water used was therefore filtrated through a 0.45 μL filter (this has shown to increase the transition temperature of PNIPAm with 0.7 °C compared to regular H₂O).[38] The reaction vessel was put in an oil bath that held 75 °C (the surrounding temperature of the reaction vessel should contain ~5 °C more than the reaction temperature because of heat loss through the vessel). A picture of this is shown in Figure 18. A stock solution of SDS was prepared by dissolving SDS in filtered deionized water (100 mL). NIPAm (1.6 mMoles) and BIS (90.8 μMoles) was put directly into the reactor and melted, before adding the SDS-solution (10 mL of 1.6 mM, 2.1 mM or 4.2 mM) using a pipette. The AAc (126 μL of 1.46 M) was put into the solution. KPS was dissolved in filtered deionized water before adding the solution (400 μL of 103.6 mM) to the reactor. The reaction was allowed to run for 3 hours. The polymer solution was poured into a pre-washed dialysis tube and put to stirring dialysis overnight.
3.3.3  Dialysis

3.3.3.1  The reason for dialysis
Dialysis is a way to clean the polymers and remove unwanted molecules/atoms.[55] Dialysis was used in this study because it is a fast and easy method of cleaning. In dialysis the polymer solution is placed in a dialysis tube which will get rid of all the unwanted compounds (salts, monomer, initiator, etc.) except the polymer in the solution, due to its high molecular weight. This is dependent on molecular weight cut-off (MWCO) of the dialysis membrane. The polymer can then stay in the tube, and all the other compounds will diffuse out of the tube and into the water due to the difference in chemical potential inside and outside of the tube.[55]

3.3.3.2  Procedure of dialysis
A dialysis tube (MWCO 14 000) was prepared by softening in water before adding a clip-on to one of the ends of the tube. The dialysis tube was washed a couple of times with deionized water before the polymer was transferred into it. The other clip-on was placed in the other end, and the tube was then placed in a large beaker under continuous stirring. The dialysis water was changed after 1–3 hours, and left overnight.

3.3.4  Freeze-drying
The solution polymers can be freeze-dried to form hygroscopic, low density powder. Introduction of drug solutions to polymers in this state has shown high efficiency of loading and encapsulation.[13]
3.3.4.1 Freeze-drying the solution polymers
The polymer solution (~10 mL) was put into a round flask suitable for one of the inlets to the freeze–dryer. It was then cooled down by stirring the flask in liquid nitrogen for a couple of minutes until the solution became solid. The flask was placed on to the freeze-dryer until the solution was completely dry (~3 hours). The freeze-dried polymer was weighed and stored for further used. The product obtained after freeze-drying is shown in Figure 19.

![Freeze-dried PNIPAm/AAc nanogels.](image)

3.3.5 Loading
The freeze-dried polymers were introduced into a solution of paracetamol or Cytochrome C through the breathing in mechanism. The incorporation method was also tried with paracetamol as a loading mechanism. These loading mechanisms have been previously described in Section 2.6.2.

3.3.5.1 Paracetamol loaded by the incorporation method
The Paracetamol (132 μmoles) was added one hour after starting the precipitation polymerization of PNIPAm/AAc.

3.3.5.2 Breathing in of Paracetamol
The Paracetamol (10 mL of 66.2 mM) was put into a solution containing the polymer in the solid state (final conc. 2 mg/mL) and put to shaking for 24 hours before centrifugation.

3.3.5.3 Breathing in and loading study of Cytochrome C
The Cytochrome C (10 mL of 8.11 \cdot 10^{-3} \text{ mM}) was put into a solution containing the polymer in the solid state (final conc. 2 mg/mL) and put to stir for 3 hours. The solutions before and after incorporating the Cytochrome C solution to the nanogels through the breathing in mechanism are shown in Figure 20. After the incorporation to the polymers the solution turns from an iron-colored solution (shown to the left of the figure) to a white color with a pale iron-color (shown to the right).
Figure 20 – Shows the Cytochrome C solutions without the polymers (left) and with the polymers (right).

Dialysis was used for the loading study of Cytochrome C (8.11 · 10^{-3} mM). The drug was first stirred for three hours after incorporation to the polymer (2 mg/mL). The solution was put into a tube and left for 24 hours of dialysis. The solution was diluted (2 times) and the concentration of the drug was measured at time point of 0, 1, 3, 6 and 24 hours using the UV-VIS.

The loading – and encapsulation efficiency of Cytochrome C was calculated using Equations 3.4 and 3.5 respectively.

**Loading efficiency:**

\[
\frac{C^0 - (C^0 - C^1)}{C^0}
\]  

(3.4)

Where \(C^0\) is the concentration of drug/polymer solution before loading and \(C^1\) is the conc. after loading (the Cytochrome C left in the polymer solution).

**Encapsulation efficiency:**

\[
\frac{C^0 \cdot \text{loading efficiency}}{100 \cdot C_p}
\]  

(3.5)

Where \(C_p\) is the concentration of the polymer in the drug solution, and the factor of 100 in the above equation is because the loading efficiency is given in percentage.

### 3.3.6 Release

**3.3.6.1 Release of paracetamol**

The paracetamol loaded PNIPAm/AAc nanogels was placed into a flask with a stirrer and placed on a heater at 50 °C. The solution was stirred for ~30 minutes before analyzing. The release of paracetamol was calculated as shown in Equation 3.6.

\[
100\% \ - \ \text{loading efficiency}
\]  

(3.6)

**3.3.6.2 Release of Cytochrome C**

The Cytochrome C loaded PNIPAm/AAc nanogels was placed into a pre-washed dialysis tube and left in a large beaker. The beaker was equipped with a magnet and placed on a magnet stirrer that maintained 39 °C. The solution was diluted (2 times), analyzed in the UV-VIS and the release was measured at different time points.
The release of Cytochrome was calculated as shown in Equation 3.7. The amount released was calculated relatively to the amount loaded, shown in Appendix B.

\[
\frac{\text{Amount released}}{\text{Amount loaded}} \cdot 100\% \tag{3.7}
\]
4 Results and Discussion

4.1 Re-crystallization of NIPAm
The nanogels used to load the drugs were synthesized from the re-crystallized monomer. The first monomer batch used had already been re-crystallized, but more was required. A monomer batch has therefore been re-crystallized during this study. The size difference of the synthesized nanogels (using the two different batches of the monomer) under identical conditions are negligible (~2 % below VPTT and ~7 % over). Similar variations have also been observed during repetitive measurements of the same solution.

4.2 Synthesis of PNIPAm
The original procedure of synthesizing PNIPAm given by Tam et al. has been modified. The mole ratio of the components has been changed; The NIPAm concentration has been slightly increased from the original 0.130 M to 0.149 M.

The particle size of the nanogels should ideally be ~50–200 nm to avoid the elimination routes in the body (Section 2.2.1) and due to that this size range is favored for intravenous drug delivery.[16] Small sizes of the nanogels have many advantages, such as avoidance of early clearance and easier modifications compared to larger networks (Section 2.9.1). This is why the size of the nanogels was tried to be optimized.

In order to decrease the particle size the SDS concentration has been increased from 0.4 mM to 1.6, 2.0 and 4.0 mM. In the presence of the ionic surfactant, the precipitation polymerization has shown to create nano-sized particles as also observed by Hendrickson et al.[16] This is due to the fact that the surfactant decreases the probability for particle aggregation (the gel growth occurs mainly through monomer or oligomer addition). The stabilizer at high temperature favors small particles. Manipulating the temperature has therefore been tried in this study. An attempt to synthesize small particles has been to ramp up the reaction temperature. An increase in temperature will compensate for the decreasing propagation rate when the monomer is consumed and increase the reaction kinetics.[16] This concurs with a study done by Lyon et al. where it was raised from 45 to 65 °C.[21] In the present study, the starting reaction temperature was set to 50 °C. This lowered reaction
temperature reduces the oligomeric radical concentration which lowers the abundance of collapsed nuclei. This favors the particle growth mechanism without appreciable nuclei aggregation because it is unlikely for bimolecular termination of two radicals on different nuclei. In addition, the concentration of the monomer is highest right after the initiation. The lower nuclei concentration combined with the high monomer concentration gives a higher propagation rate than initiation rate. This will give growth of nuclei with same speed in early polymerization stages. However, the propagation rate will decrease since the monomer concentration decreases. Due to this the temperature is thereafter raised.[21] The temperature in this study was increased to 80 °C after an hour. This gives an increase in the decomposition of persulphate and generates more radicals. When the temperature is increased, the monomer concentration is low and the growth on the nuclei/precursor particle is favored over nucleation because of decrease in monomer concentration and stronger vdw-forces between nuclei and/or precursor radicals than the forces before the nucleation stage. The sulphate end-groups create electrostatic repulsion and the particles are stabilized from coagulating while still capturing oligomeric radicals and unstable nuclei, and no secondary nucleation from unstable nuclei are expected to be formed. A similar trend has been observed by Lyon et al. in their study of microgels.[21]

This ramping of the reaction temperature gave smaller size of the particles in this study. However, the heater makes it difficult to get uniform reaction temperature when increasing it since it takes time before it stabilizes at these elevated temperatures. The reaction temperature was therefore set constant at 70 °C. This was also considered reasonable since the easiest and most effective way of decreasing the particle size was shown to be the increase of the surfactant concentration during synthesis.

4.3 The effect of the surfactant
The decrease in particle size with increasing surfactant concentration is shown in Figure 21 as a function of temperature to illustrate the influence of the SDS concentration.
The reason for the decreasing size with increasing SDS concentration is due to the early stabilization of the polymer by the surfactant.[44] However, it should be mentioned that when PNIPAm/AAc was left for dialysis and measurements were done at different time points, the particle size increased at the later time points. This has been proven by differences from newly synthesized polymers to a day of dialysis (4 % difference under VPTT and 16 % over) were not as significant as to eight days of dialysis (43 % difference under the VPTT and 3 % under). This implies that the surfactant was (weakly) bound to the nanogel, and thereby compressed it. When the solution was left over a longer period of time, a higher concentration of the surfactant had probably diffused through the dialysis tube. The polymer thereby had more interactions with water and the possibility to be more swollen, thereby the increased particle diameter. However, when over the VPTT the particles will be de-swollen and exhibit almost the same morphology (and thereby almost the same size). This also explains smaller differences, when over the VPTT for the solutions at different time points in dialysis.

As mentioned, the surfactant concentration has been increased to optimize the size of the nanogels. At high conc. of the SDS (5 and 5.5 mM) the polymer solution became visible by a blue reflection as shown in Figure 22. This color did not disappear after dialysis, which supports the assumption of bound SDS to the nanogel network.
Since it was difficult to quantify the surfactant concentration inside the dialysis tube, a phosphate–buffer was used as an attempt to get rid of all the surfactant molecules. The buffer was used due to the assumption that it could act as an ion exchanger: The potassium–ions can replace the sodium ions of SDS. The principle of this is shown in Figure 23. The potassium has higher affinity towards the de-protonated hydroxyl group of AAc due to the charge density mapping between COO$^-$ and K$^+$. The charge density mapping of these ions will be higher than for COO$^-$ and Na$^+$. It is much easier for the outer shell electrons of K$^+$ (than Na$^+$) to be shared with the negative oxygen center.

![Figure 23 – Sodium bound to the AAc can be replaced by potassium ions of phosphate – buffer.][58]

The phosphate–buffer was made by tuning a solution of K$_2$HPO$_4$ (50 mM) and KH$_2$PO$_4$ (50 mM) to pH 7.4. The water in the beaker was replaced by the buffer and the polymer solution in dialysis tube was put to stir in it. After an hour of dialysis, the polymer solution seemed to have diffused out of the tube. This confirmed the assumption that potassium can replace the sodium of SDS. Thereby the surfactant molecules alone were able to diffuse out of the tube, and the original water solution in the tube was replaced with the buffer. This also explained the change of color in the tube; the solution turned from its normal turbid white color to transparent. In addition, much less fluid was observed, which can imply that K$_2$HPO$_4$ and KH$_2$PO$_4$ replaced the larger SDS molecules. The solution in the tube was analyzed in the DLS, and no particles were present. This implied that the nanogels probably degraded in the absence of the surfactant.

### 4.4 Characterization of the nanogels

#### 4.4.1 Stability and dilution of the nanogels

When measuring the particles using the DLS, three parallel measurements were taken. The solutions at elevated temperatures were allowed to stabilize at the given temperature for few minutes before measuring.
4.4.2 The cuvettes
The size cuvettes (glass cuvette 6G and plastic cuvette) and the zeta potential cuvette used in the DLS are given in Figure 24.

![Image of cuvettes used in DLS](image)

*Figure 24 – The cuvettes used in the DLS: The Zeta Potential cuvette (left), the glass size cuvette (in the middle) and the plastic size cuvette (right).*

The differences in size measured can be seen in Figure 25 (the nanogels were synthesized with 2 mM SDS, and measured before dialysis), which illustrates slightly larger size in the glass cuvettes (shown as number 2). These size differences are not of importance due to small changes measured in size by the sample in the same cuvette as well. In addition, at the elevated temperatures there are no significant differences between the sizes. The glass cuvette was chosen due to slightly lower polydispersity index (PDI). The low PDI of the particles has also been observed by Hendrickson et al. for core/shell PNIPAm/AAc hydrogels.[16]

![Size comparison graph](image)

*Figure 25 – Shows the size difference as a function of a plastic (1) and glass cuvette (2) at 25 °C and 45 °C.*
4.4.3 The size of the nanogels

4.4.3.1 Freeze-dried polymers

The DLS was used to establish possible size- and PDI differences from the original polymer solution and the freeze-dried polymers (1 mg/mL). The difference between before and after freeze-drying the solution is shown in Figure 26 (these nanogels were synthesized with 2 mM SDS).

![Graph showing size differences before and after freeze-drying](image)

Figure 26 – Shows the difference in size between polymers before freeze-drying (1) and after (2) at 25 °C and 45 °C.

The differences in particle size before and after freeze-drying are < 4 %, which is considered insignificant (due to observation of these size changes also in the same solution). However, the PDI before and after introducing the polymers into the solid state has decreased at room temperature. This can be due to the fact that the particles swell more uniformly when introduced to the solution in the solid state, rather than under the synthesis of the polymers. All measurements in the DLS were continued with the freeze-dried polymers at concentration of 1 mg/mL.

4.4.3.2 Particle size as a function of time in the solid state

Freeze-dried polymers (8 % BIS, 2 mM SDS) were kept in the solid state. However, differences were observed when the polymers were left in this state and analyzed at two different time points (after a day and after a week). Differences in size were observed both at 25 °C and 45 °C as shown in Figure 27. This is probably due to less stability of the polymers in the solid state. The nanogels will be more stable in solution, and should thereby not be held in the solid state for a longer period of time. This is also confirmed by higher PDI after a week in the solid state.
4.4.3.3 The particle size as a function of the surrounding temperatures

It is also worth mentioning that the particle size could show different values dependent on the surrounding temperature. The solution could for example show significant difference in particle size when the solution was held at ~3 °C compared to the same solution at room temperature. This was tested and is shown in Figure 28 for a freeze-dried solution. Differences were observed both over and under the VPTT. The larger particles were observed in the cold solution. This was also proven by Samah et al. who observed that the size of the particles expand when it is cold (refrigerator cold: 2-4 °C) compared to particles in solution at room temperature.[59]
Figure 28 – Shows the collapse of the polymers when the solution is kept at room temperature (1) and at ~3 °C (2).

The ideal size of the particles should be in the range of the PNIPAm/AAc hydrogels that Choi et al. synthesized; ~200 nm.[8] This was also achieved in this study at certain conditions. However, different environments have given different sizes of the hydrogels. The same solution temperature is therefore important, and the solution should be held at room temperature ~1 hour before performing measurements.

4.4.4 The VPTT

DLS was used to measure the size of the nanogels as a function of increasing temperature. This study was performed with two batches of freeze-dried nanogels in solution (1 mg/mL), as can be seen in Figure 29. An interesting observation is that the PDI decreases at elevated temperatures. This is because of the fact that the particles can be swollen to different degrees when below VPTT. At elevated temperatures the morphology of the de-swollen particles will be more similar. The decrease in size as a function of temperature with particles synthesized under slightly different conditions is shown in Figure 29. The filled circles represent particles synthesized with 5 % BIS and 4 mM SDS, while the unfilled color represent synthesis with 8 % BIS and 3 mM SDS. The latter particles have smaller size, and this is probably due to increased BIS concentration as this has shown to be able to decrease the size. This can be due to smaller hydrodynamic size when increasing the cross-linking within the particle. In addition, since the difference in the SDS concentration added during synthesis is not that significant, the cross-linker is believed to change the size between these two solutions as discussed in Section 2.2.3.
The decrease in size of the particles with 4 mM SDS (filled) goes over a wide temperature range, which can be due to higher particle size as it takes more energy (heat) to collapse a larger network. The size decreases continuously from 30 °C when increasing the temperature up to 47.5 °C. The same continuous trend has been observed by Choi et al. This group achieved narrow size distribution below and above VPTT. They synthesized PNIPAm/AAc particles and got VPTT at 37 °C. They got particle size of ~200 nm, which shrank to below 100 nm over a temperature range of 27–40 °C. This de-swelling achieved over time range is most probably due to the incorporation of AAc.[8] These results by Choi et al. are similar to the observations in this study. This kind of decrease can help avoid the entrapment of possible encapsulated drug inside the nanogel. The continuous decrease will according to Samah et al. give more release efficiency, compared to a post-collapse of the particles when exposed to heat.[59]

Since there is no abrupt phase transition it was difficult to set an exact VPTT. The calculated VPTT was therefore based on the temperature the network starts to collapse and the temperature at which collapse no longer occurs. The calculation of VPTT for the filled circles in the figure above is shown in Equation 4.1. The VPTT of the polymeric network was calculated to be ~39 °C.

\[
30 \, ^\circ \text{C} + \frac{47.5 \, ^\circ \text{C} - 30 \, ^\circ \text{C}}{2} = 39 \, ^\circ \text{C}
\]  

The second solution (unfilled) was synthesized with less surfactant, in addition to higher cross-linker (8 % BIS, 82.3 % PNIPAm and 9.7 % AAc). The smaller size explains the more abrupt phase transition of the polymer, since there are smaller networks to collapse. Due to this abrupt collapse more time points were taken during the steepest decrease in size. Curve fitting was used to calculate the VPTT as shown in Appendix C. The VPTT was calculated to
be 36 °C. This VPTT was close to the VPTT achieved by Choi et al. which synthesized the similar hydrogels.[8]

4.4.5 The reversibility of the hydrogel network
When the temperature was increased to above VPTT and then decreased to below, the nanogels did not show reversibility in the previous work done at Ugelstad Laboratory.[57] However, in this study the reversibility of the freeze-dried nanogels (1 mg/mL, 8 % BIS, 2 mM SDS) was confirmed. This study is shown in Figure 30. The solution was first heated to 55 °C before cooling down to 25 °C, which showed approximately the same size as before heating. In addition, the trend of the swelling and de-swelling was also observed to be the same. The reversibility has also been confirmed by Lewis et al. who synthesized polymers consisting of NIPAm - (2-methacryloyloxyethyl phosphoryl-choline) – NIPAm triblocks. These polymers have shown some hysteresis when cooling down.[60] The hysteresis observed in Figure 30 is insignificant since the differences in size have also been observed from the same solution when measuring twice. This makes the nanogels synthesized in this study very interesting in drug delivery applications. This behavior of the nanogels is an advantage when considering them as drug carriers. The interactions between the desired cargo and these networks will not change upon heating, which gives possibility for on/off switch, and thereby more controlled release.

![Figure 30](image.png)

**Figure 30 –** Shows the reversibility of the hydrogel networks. The size is plotted as a function of temperature when increasing the temperature (filled circles) and decreasing the temperature (unfilled circles).

4.4.6 The size of the particles as a function of temperature at high and low pH
The particle size as a function of pH was also measured at different temperatures. The pH was first tuned to 9 and decreased to 3. Since the particles showed almost completely reversibility this should not affect the results at pH 3. This has been done for a freeze-dried polymer
solution (1.6 mM SDS) and is plotted in Figure 31. At pH 9 the size of the particles did not show any significant differences due to the inhibited chain collapse of the particles caused by Coulombic repulsion of the de-protonated hydroxyl groups. The strong repulsive forces of the shell dominate and this causes decrease in the average inter-chain distance in the core as stated by Hendrickson et al. with the study of PNIPAm/AAc core/shell hydrogels.[16] This is the reason of the retarded collapse of the particles (Section 2.3.4).

The particle size at pH 3 increased when increasing the temperature. This can be explained by the fact that the particles are in a hydrophobic state at low pH. When over the VPTT, the hydrophobic interactions are strongly dominating, and attraction between the particles will occurs causing aggregation. This assumption is also supported by observation of the increased PDI when increasing the temperature.

4.4.7 The zeta potential

4.4.7.1 Variation of the zeta potential with pH and temperature

The zeta potential for the same polymer solution at pH 3 and 9 were also measured. This observation is interesting as the zeta potential has a direct connection with the actual gel charge density (in addition to the degree of surface charge) and particle topology (“hairiness”) as studied by Lyon et al.[18] The zeta potential for the different pH-values is shown in Figure 32 as a function of temperature. The solution was tuned to pH 9 with NaOH (< 0.1 M) before adding HCl (0.1 M) tuning the solution to pH 3. The zeta potential for pH 9 is approximately the same both before and after increasing the temperature. This concurs with the small change in particle size due to the dominating repulsive forces which is observed by Hendrickson et al.[16]
The zeta potential before and after increasing the temperature above VPTT is on the other hand significant for the polymer solution at pH 3. The potential decreases when the temperature is increased. At room temperature, the polymers will be protonated, favoring the inter-molecular forces. The negative surface charge was therefore absent and approximately neutral zeta potential was observed. When increasing the temperature the particles are assumed to be aggregated, thereby the increased particle size and PDI.

The study showed that the repulsive forces are highly dominating at pH 9 and that the particles are very hydrophobic at room temperature at pH 3. This was confirmed by both the size of the particles and the zeta potential as shown in Figure 31 and Figure 32.

4.4.7.2 Variations of the zeta potential caused by the surfactant

The zeta potential for the polymer-solution with different SDS concentration was another interesting observation, shown in Figure 33. The zeta potential is given as a function of the SDS concentrations used during the synthesis of PNIPAm/AAc with very low concentration of the surfactant (0.4 mM) (concentration used by Tam et al.) [56], and two different concentrations used in the study (2 mM and 4 mM) at 25 °C, and 50 °C. The decreasing zeta potential as a function of increasing SDS concentration confirms that the surfactant has not been completely removed during the dialysis. The surfactant will contribute towards the negative surface charge and thus towards lower zeta potential when increasing the concentration of the surfactant. The three samples have an increased zeta potential when increasing the temperature (~16 %). This is due to the hydrophobic interactions when the particles have collapsed as described in Section 2.3.2. The surface charge density has increased as stated by Choi et al.[8], but particles will make inter-molecular interactions and probably entrap the surfactant inside the polymeric network. This will contribute to more H⁺-ions in the solution.
The increased zeta potential as a function of the SDS conc. and temperature should be noted since this can influence interactions that the nanogels make. However, the heating effect on the zeta potential was lowered after the particles were freeze-dried. In addition, the potential increased as can be seen in Figure 34. The zeta potential is given as a function of the nanogels before freeze-drying and the nanogels freeze-dried in solution (1 mg/mL) at 25 °C and 50 °C. Both solutions were synthesized under the same reaction conditions with SDS concentration of 4 mM. However, since the zeta potential decreases after the freeze-drying this can indicate that the SDS molecules is removed during the freeze-drying (which concurs to the previous assumption that the zeta potential is affected by the surfactant). This assumption also explains why the zeta potential is approximately the same (~7 % change) before and after increasing the temperature. However, the freeze-drying did not show any significant effect on the size of the nanogels. This is most likely due to different concentration in the solutions before and after freeze-drying because the polymer swells better in more dilute solutions.
Due to the observation that the zeta potential of the freeze-dried polymers did not vary that much over and under the VPTT, the pH was measured for freeze-dried polymers in solution with different SDS concentration. The pH variations in Figure 35 are considered insignificant as the change is ~10%. This observation implies that the AAc is more exposed after removal of SDS, which is also supported by the observation of pH 4.9 in the polymer solution before freeze-drying. The freeze-dried polymer’s interactions are therefore not to be influenced by the surfactant.
4.5 Loading and release studies

4.5.1 Scattering polymers
The nanogels have also been analyzed in the UV-VIS to establish any scattering. This analysis revealed a possible disturbance from the nanogels. A peak at ~209 nm is observed in Figure 36. This shoulder decreased with decreasing concentration of the polymer, until it vanished. However this peak is not observed at concentrations at which the paracetamol is measured.

Figure 36 – Shows scattering of the polymer (in polymer solution with concentration of 1 mg/mL.)
4.5.2 Loading paracetamol

The scattering from the nanogels were not observed for the analysis of free paracetamol after the breathing in mechanism. This drug has been studied due to that it is a standard drug widely used for studying both loading and release from a wide range of polymeric nano-carriers.\[61, 62\] From initial studies with paracetamol, it has been observed that the drug shows more hydrophobic interactions than initially expected from literature. These interactions are not desired for \textit{in vivo} applications.

4.5.2.1 Calibration curve of paracetamol

A calibration curve was made for paracetamol by making a solution with known concentration of the drug. The solution was adequately diluted to have absorbance values with the linearity range of Beer-Lambert’s law, as shown in Section 3.2.2.1, at 243 nm. These concentrations and the corresponding absorbance are shown in Figure 37. A linear regression was performed as shown in the figure that gives the absorbance as a function of the concentration.

![Figure 37 – Shows the calibration curve for paracetamol.](image)

This curve was used to calculate concentration of free paracetamol in solution. The drug incorporated hydrogels were placed into centrifuged at 14 500 rpm for 15 minutes using centrifugal filters (MWCO 15 000). The filtrate was diluted (700 times) and analyzed using the UV-VIS.

4.5.2.2 Loading of paracetamol during the synthesis of the polymer

Paracetamol has been added during the synthesis of PNIPAm/AAc. According to Chakraborty \textit{et al.} this incorporation method has shown to be able to load more drug compared to the
However, no peak in the UV-VIS for the drug was observed but a shoulder as shown Figure 38. This shoulder was observed where the paracetamol peak usually occurs. This shoulder could be due to scattering from the polymer. However, the shoulder did not disappear when the incorporated polymer solution was filtrated (MWCO 5000). The filtrate should show a peak for the drug only, due to the large molecular weight of the polymers. It is however possible that the shoulder is from the nanogels since they can pass through the filters in the collapsed state: When the drug is incorporated during synthesis there is a change in the relaxation dynamics of the polymer. The polymer will thereby have smaller size and may pass through the filter. This method has only been tested in this study, and since no quantification of paracetamol could be calculated, the main mechanism used for loading was the breathing in mechanism.

![Figure 38](image.png)

*Figure 38 – Shows a shoulder where the peak of Paracetamol is usually observed.*

### 4.5.2.3 Centrifugation

To quantify the paracetamol breathed in to the polymers, centrifugation has been utilized. The tubes used contained filters which are illustrated in Figure 39.

![Figure 39](image.png)

*Figure 39 – Shows centrifugation tubes with filters.*

Eppendorf tubes have also been tested. However, these tubes did not get rid of the polymer from the supernatant: When analyzed using DLS, nanogels could be observed. A collapse has also been observed, but with a smaller magnitude. This can be due to some structural changes
of the polymers when centrifuging. This excluded the centrifugation tubes without the filters for analysis of incorporated paracetamol.

The filters in the centrifugation tubes were changed to filters with known MWCO (from 5000 to 3000 Da). This was because paracetamol is a small molecule with molecular weight of 151,163 g/mol, and it was assumed that the filters would allow free passage of paracetamol into the filtrate solution, while the polymers would be captured by the filters. A possible error that could occur was drug entrapped in the filters, blocked by the polymers. This could be a possibility since the nanogels get stuck in the filter, and the drug has to pass through these networks. It is therefore possible that the nanogels bind the drug when it is forced down to the filters during the centrifugation. This has been tested and the paracetamol concentration was approximately the same before and after centrifugation, which excluded this assumption as a possible error. This observation was compared with the filtrated drug and the drug in the polymer solution. The drug concentration had the same concentration before and after introducing the drug solution to the polymers. The disturbance from the polymers was not observed, which implied that the drug molecules “hid” the less polymer molecules. This assumption is difficult to confirm with the UV-VIS. However, this is of no importance for this study as the paracetamol was quantified. The importance of this observation was that after loading the filtrate showed less drug conc. than in the drug/polymer solution, which implied that there had been loading.

Most of the studies of paracetamol have been used with MWCO 5000, while the filters with MWCO 3000 were used for pH-study of paracetamol. Due to the smaller pores, the time of centrifugation had to be increased to 40 minutes. This has not showed to have an influence on the concentration of drug when measuring the concentration before and after centrifugation. These observations have been done before and after loading, and thereby the breathing in mechanism has been chosen as the loading mechanism used for the loading studies.

4.5.2.4 Loading with the breathing in mechanism

After the filtration, the paracetamol in the filtrate has been analyzed in the UV-VIS and the concentration has been calculated using the calibration curve shown in Figure 37. Parallel loading batches were analyzed, and loading of paracetamol (66.2 mM) has been observed as shown in Figure 40. However, it should be mentioned that the loading has not been stable, as some of the parallels showed no loading. This could be explained by the assumption of a more hydrophobic drug. If the drug reaches interactions with hydrophobic sites when the polymer is swollen there may be loading. This loading will be random and may not always occur. This assumption is supported by the higher and more stable loading at elevated temperature as shown in the figure below. This was proven for these parallels, in addition to the parallels that showed no loading.

Both parallels given in Figure 40 were heated at 45 °C (~1 hour). However, two different dilution factors were used when heating the solution: 5 (for the solution with 0.4 mg/mL polymer) and 10 (for the solution with 2 mg/mL polymer). This implies the loading is not as efficient in diluted solutions. This is in agreement with the fact that a concentrated solution is needed in when utilizing the breathing in mechanism, which is a highly effective loading method.[13] It is therefore not possible to compare these solutions. It is however interesting to
see the trend of the increasing loading – and encapsulation efficiencies after heating. This implies that paracetamol is more hydrophobic than initially assumed.

*Figure 40 – Shows the a) loading efficiencies in percentage and b) encapsulation efficiencies in mg drug/mg polymer of the paracetamol–loaded polymers at 25 °C and 45 °C.*
4.5.2.5 Optimizing the paracetamol – and polymer concentration

The efficiencies of loading have been tried to optimize with the polymer - and drug concentration as shown in Figure 41.

Figure 41 – Shows the (a) loading efficiencies in percentage and (b) encapsulation efficiencies in mg drug/mg polymer as a function of the concentration of drug (filled circles) or polymer (unfilled circles).

The encapsulation – and loading efficiencies were calculated with Equation 3.1 and 3.2 in Section 2.6.2 and an example of these calculations is given in Appendix B.
Three drug concentrations have been tested: 16.5, 33.1 and 66.2 mM at constant polymer conc. (0.4 mg/mL). The loading efficiency was slightly higher for 33.1 mM drug, but since the encapsulation efficiency was significantly higher for the highest drug concentration this has further been used.

The polymer concentrations tested were 0.4, 0.8, 1.6 and 2 mg/mL with constant paracetamol conc. (33.1 mM). The lowest polymer concentration has shown significantly higher encapsulation efficiency, while the highest polymer concentration has shown significantly higher loading efficiency. Both concentrations have been used in the initial paracetamol studies. However, the highest polymer concentration has been chosen in further studies due to the need of high enough polymer concentration if multiple drugs are desired in the network. More available sites will be required in these studies, which is relevant for studies of targeted drug delivery systems.

The loading – and encapsulation efficiencies achieved show the trend of loading, it is however important to note that the parallels did not show the same efficiencies as pointed out in Section 4.5.2.4. The assumption of randomly hydrophobic interactions with paracetamol and polymer also concur with the observation of lower loading – and encapsulation efficiencies when repeating a loading experiment. This can be seen by comparing the two parallels shown in Section 4.5.2.5 and the figure above (66.2 mM drug and 0.4 mg/mL polymer).

The aim of these observations has been to optimize the concentrations, which has made the quantification of the loaded drug less important. The interesting observation has been the concentrations that have given the highest efficiencies, which has been used in the further studies described below.

4.5.2.6 Loading at elevated temperature
The optimized polymer (2 mg/mL) and drug (66.2 mM) concentrations have been used when trying to load at elevated temperature. This loading study has been performed due to the observation of higher and more stable loading when over the VPTT. The drug was introduced to the polymers at 50 ºC and shaken for 24 hours at 37 ºC. Two loading methods were used: Introduction of the solid drug to the polymer and the solid polymer introduced to the drug solution. Both methods seemed to load the polymer. An encapsulation efficiency of 2.7 mg drug/mg polymer and a loading efficiency of 53-54 % were achieved with both methods. These efficiencies are given in Figure 42. The loading efficiency had a lower value compared to the efficiency achieved by Lyon and Smith (93 %). They used the breathing in mechanism to entrap siRNA to Poly(N-isopropylmethacrylamide) nanogels. The encapsulation efficiency was on the other hand significantly larger compared to the same study (16 μg siRNA/mg polymer).[13] This could be due to different polymer or/and drug used, or the duration of the loading.

The loading – and encapsulation efficiencies have been confirmed to increase at elevated temperatures, which support the assumption of hydrophobic interactions between paracetamol and the polymer.
Figure 42 – Shows the a) loading efficiencies in percentage and b) encapsulation efficiencies in mg drug/mg polymer for paracetamol – loaded polymers after loading at elevated temperature. The bar to the left represents the loading study when introducing the drug to the polymer solution and the bar to the right when introducing the polymer to the drug solution.

4.5.2.7 Loading with decreased pH
Since paracetamol was more efficiently loaded at elevated temperatures, it was also assumed a similar trend at lower pH when the hydroxyl groups of AAc are protonated as stated in Section 2.6.5. At lower pH the AAc will be highly protonated as the pKₐ-value of the acid is 4.25 as described in Section 2.3.2. The drug loaded polymers were thus tuned to pH 3 by HCl (0.1 M). The concentration of bound paracetamol is given as a function of the corresponding
conditions in Figure 43. The condition represents the pH and how long the solution has been stirring at the given pH.

The measurement at pH 4.61 (normal pH of the polymer/paracetamol solution as both polymer and drug influence the pH) the *breathing in mechanism* has been used (with 66.2 mM paracetamol and 2 mg/mL polymer). As can be seen from the figure; after introducing the drug to the polymers by shaking the solution a couple of minutes some loading has been observed at normal conditions. This could imply that loading of 24 hours is not necessary to load the polymers, but this needs to be further investigated. However, more loading has been observed when decreasing the pH. After 30 minutes of stirring the solution at pH 3 has shown a loading efficiency of almost 60 % and an encapsulation efficiency of 14.1 mg drug/mg polymer. These efficiencies cannot be completely compared with the efficiencies achieved when raising the temperature to 45 °C in water due to the dilution factor used. The efficiencies achieved when loading at higher temperature are on the other hand comparable, but the encapsulation efficiency achieved by decreased pH is significantly higher. It should be noted that the nanogels did not completely collapse at the loading at elevated temperatures, and higher loading may be achieved by increasing the temperature further (for example to 45 °C).

These loading studies suggest that hydrophobic interactions exist between the drug and polymer. These observations made it very interesting to study the nature of the release from the network.
Figure 43 – Shows the a) loading efficiencies in percentage and b) encapsulation efficiencies in mg drug/mg polymer of the paracetamol-loaded polymers. These efficiencies are given as a function of pH 4.61 (the filtrate before adjusting pH), pH = 3 (right after adjusting the pH) and pH 3 after 30 minutes of stirring the solution at pH 3.
4.5.3 Release of paracetamol

4.5.3.1 Release after loading at elevated temperature

The release of paracetamol has been studied after loading at elevated temperature. The solution has been heated further to 70 °C which has given release as shown in Figure 44. The calculation of the release is given in Appendix B. This observation implies that the breathing in mechanism gave slightly more efficient release compared to when the drug was loaded by introducing it to the polymer solution.

The loading has been at a temperature where the nanogel can make hydrophobic interactions (37 °C). It is therefore assumed that the drug makes hydrophobic interactions with the polymer when loaded and as the temperature has been further increased to far beyond the VPTT; the drug is squeezed out as explained in Section 2.7.1. The release has also been observed when the polymers with the incorporated drug (done by the breathing in mechanism) have been left at room temperature. This has shown 85 % release (of the loaded drug) after three days. This concurs with the loading studies at room temperature where the polymer had lower loading (when it is more hydrophilic). The release from the nanogels in the hydrophobic state has therefore been assumed to be triggered when the nanogels become hydrophilic.

![Figure 44](Image)

Figure 44 – Show the percentage release of incorporated drug from the hydrogels, loaded with two different methods: Incorporation of solid drug to the polymer solution and the breathing in mechanism.

4.5.3.2 Release after loading at pH 3

After the loading at pH 3 the pH has been tuned to 7 with NaOH (0.1 M). Release of 46 % of the loaded drug has been observed after an hour at pH 7 (calculation of release in Appendix B). At this pH the polymers are de-protonated and they are hydrophilic. This implies that the release can be triggered by a change of the polymer’s hydrophobicity. However, this is not
desired when introducing the drug incorporated nanogel to the body; the drug will be released already in the blood stream and the targeting will not be specific.

The release of paracetamol has been confirmed when the nanogels are hydrophilic. Random loading has been confirmed at the same state in the loading studies (due to random interactions), and more efficient and stable loading have been observed when the nanogels enter the hydrophobic state. The drugs hydrophobic characteristics make it difficult to use the drug in vivo.

4.5.4 Loading of Cytochrome C
In order to ascertain the hydrophobicity of paracetamol, the biologically relevant model protein - Cytochrome C has been chosen. It being a protein not only shows pH-dependent properties but also mimics physio-chemical properties of several hydrophilic clinically relevant drug molecules like siRNA, pro-drugs and peptides.

4.5.4.1 Calibration curve of Cytochrome C
Cytochrome C was analyzed and a calibration curve was made based on the absorbance values, shown in Figure 45. The Cytochrome C concentration used was $8.11 \cdot 10^{-3}$ mM when introducing it to the polymer solution (2 mg/mL). The Cytochrome C solution has been diluted (2 times) before measured in the UV-VIS. The concentration has been calculated by the absorbance value from the calibration curve. The values are taken at 409 nm, where the heme-group of the protein is observed.

Since this drug is a protein, it was assumed that heat would affect it. Due to this assumption the drug has been heated to 50 °C. Cytochrome C solution ($4.06 \cdot 10^{-3}$ mM) has been analyzed in the UV-VIS before and after heating as shown in Figure 46. The light blue line was before
heating, and the dark blue line was after heating (50 °C). This study has discovered a difference between the abs. lines. However, the magnitude of the heme–group showed approximately the same value. The calibration curve was also assumed valid for studies performed at high temperatures.

4.5.4.2 Polymers introduced to the solution of Cytochrome C
Cytochrome C has shown a larger peak at 409 nm (where the heme–group of Cytochrome C has been identified) in the polymer solution compared to a pure Cytochrome C solution. This trend is shown in Figure 47. As seen in this figure, Cytochrome C has gained higher absorbance when it has been introduced to the polymer solution. In addition, a peak at ~209 nm has also been observed. This has complicated the calculations of loaded and released drug. However, the calibration curve has been used to calculate the concentration with the assumption of a proportional relationship between the absorbance and concentration also when the polymer is added. Examples of the calculations of bound Cytochrome C are given in Appendix B.
Another interesting observation has been that the Cytochrome C peak shifted magnitude at different dilutions. Higher dilution factor gave larger abs. value (30 % when doubling the factor from 3 to 6). The reason for this phenomenon is due to different interactions between polymer-protein and polymer-water at different concentrations when adding different amount of water. The dilution factor has therefore been kept constant (2 times dilution) throughout the study of the protein.

4.5.4.3 Dialysis

As expected, a higher absorbance value at 409 nm has been observed for the more diluted filtrate. More dilution eas required due to small volumes of the filtrate. The filtration could therefore not be performed with Cytochrome C. In addition, Cytochrome C (12 327 g/mol) is a prominent larger molecule than paracetamol (151,163 g/mol) [24, 64], and a separation method with filtration could not be performed. This was due to that the heme-group in the filtrate has been observed in the UV-VIS, and the color of iron in the filter. These observations implied that the protein had been degraded. It was therefore difficult to quantify the amount of Cytochrome C in the filtrate.

Since the filters could not be used, the drug was tried centrifuged without filters (Eppendorf tubes). The Cytochrome C supernatant had the same concentration as before centrifuging. However, the polymers were observed in the supernatant. This has also been observed previously when centrifuging the polymers. Since the Cytochrome C has been assumed to be bound to the polymers there has been suspicion that the interactions between drug and polymer would create larger molecules and be driven out of the supernatant. This did not occur and these observations excluded study of loading and release of Cytochrome C through centrifugation. The studies with Cytochrome C have therefore been performed through dialysis (MWCO 14 000). The diffusion of free Cytochrome C has been confirmed by complete diffusion of a pure solution of Cytochrome C from the dialysis tube within a time study of 24 hours. Assumption taken from this is that all free Cytochrome C diffuse out of the
tube after 24 hours. The Cytochrome left in the solution was therefore bound to the high molecular weight polymer.

4.5.4.4 Loading
The dialysis has been used for the loading study, which is a new method of quantified loading. Different time points have been taken and the loading has been calculated differently than for paracetamol due to that bound drug has been analyzed. An example of the calculations of loaded Cytochrome C is given in Appendix B.

According to the assumption that all of the free Cytochrome had diffused out in 24 hours the last time point has been taken after 24 hours. These time points are given with the decreasing concentration of the drug in Figure 48. The incorporated polymer used was synthesized with 2 mM SDS.

The concentration of Cytochrome C decreases from the time point 0 to 6 hours. Thereby it can be assumed that most of the free Cytochrome C already had diffused out during the first hours of dialysis since it is hydrophilic. The concentration differences between the deionized water outside the tubes and drug solution with the polymers (synthesized with 2 mM SDS) in the tube was more significant at the start of the dialysis and more of the drug is thereby forced out through osmosis caused by higher concentration gradient. The loading – and encapsulation efficiencies of Cytochrome C in this study has been calculated to be 85.6 % and 0.167 mg drug/mg polymer respectively.

![Figure 48](image.png)

*Figure 48 – Shows the Cytochrome C conc. as a function of time in loading study performed through dialysis.*

After confirming successful loading of Cytochrome C through dialysis, the release kinetics of the drug was ready to be studied.
4.5.5 Release of Cytochrome C

The release studies have also been performed through dialysis, but at elevated temperature (39 °C) and different pH-conditions. The release in percentage of the loaded drug is given as a function of time in Figure 50 a. The first 24 hours of the study is especially interesting when considering the drug for in vivo applications. The time points between 0 and 24 hours are shown in Figure 50 b. Three release studies have been performed; at normal pH-condition, pH 3 and in PBS-solution. The study at pH 4.4 (natural pH of Cytochrome C-polymer solution) has been done with polymers synthesized with 2 mM SDS and 8 % BIS, while the pH 3 – and PBS study has been done with 4 mM SDS and 5 % BIS. The difference between the studies should be noted. However, the results should show the same trend since it is expected that the polymers should possess comparable properties.

The release studies revealed a clear peak shift, which has been clearly observed at the last time point taken in the release studies (75 hours). This is shown in Figure 49. The peak shift was due to that more water had entered the dialysis tube after many hours in dialysis. Thereby the polymer-water and polymer-drug interactions differed. The peak shift was at ~9 nm (from 409 to 398 nm). The absorbance value used has been taken at 409 nm also after 75 hours.

![Figure 49](image_url)

*Figure 49 – Shows the peak shift after 75 h of dialysis (light blue) compared to the peak after 1 h (dark blue) for Cytochrome C.*

The release has shown to be continuous at pH 4.4 with 22 % release after 24 hours. The release rate is believed to increase if the temperature is increased. The nanogels were not completely collapsed at 39 °C and not all of the drug solution is squeezed out of the network.

The release study at pH 3 has shown a fast decrease of the drug concentration. After 3 hours the release was already 13 %, which was higher for both releases at normal pH conditions and in PBS. The release was 24 % after 24 hours. From this study it should be noted that the first time point (1 hour) has shown a higher value of the released drug compared to the two next time points. This was most probably a measurement error.
The release study in PBS was continuous from time point 1 to 3 hours. More time points have been taken in the beginning of this release study due to possibility for fast release. This has been proven opposite: The release has been slow and not as much drug has been released compared to the other two release studies as can be seen in Figure 50.

Figure 50 – Shows three different release studies of Cytochrome C after (a) 75 h and (b) 24 h. The release studies are done in PBS (filled circles) and at pH 4.4 (triangles) and pH 3 (unfilled circles).
This study has been ended after 24 since it interesting to notice the release in the first hours when the concentration gradient for diffusion is highest for in vivo applications. In addition, most of the drug should be released already after 12 hours in PBS.[13] This is an assumption that has been taken from a study by Smith et al. They used the breathing in mechanism to load the nanogels and a certain concentration of drug was released before 12 hours at 39 °C in PBS. The group achieved a retention of ~67 %, which meant efficient entrapment of the oligonucleotide in the nanogel. This was an important quality discovered since it could be compared to the time needed to extravasate into a tumor by EPR. After 35 hours a large fraction of the drug was retained.[13]. The retention achieved by Smith et al. is slightly lower than the observation in this study. The retention has been observed to be 89 % (11 % release) after 24 hours. These release rates should be comparable since the drugs used are bio-macromolecules. However, differences could be due to different polymers that have been used: Poly(N-isopropylmethacrylamide networks compared to PNIPAm/AAc networks can make different interactions with the drug.

These observations have proven that the release of Cytochrome C is more efficient at low pH, which is where the hydroxyl groups of AAc are highly protonated as stated by Bysell et al. (Section 2.6.5). [9] That is why it would be interesting to see if the release could be increased by increasing the temperature, which probably forces the nanogels further to the hydrophobic state.
5 Conclusion
The PNIPAm/AAc nanogels were synthesized under different reaction conditions in order to optimize the size. Once the optimization was achieved, nanogels that showed around 50% collapse above VPTT were used for loading and release studies. DLS measurements confirmed the reversibility in the collapse of the nanogels, indicating their application for controlled release with a capacity to store the cargo over prolonged time. The temperature – and pH sensitive responses exhibited by these nanogels are ideally suited for loading and release studies of biologically relevant molecules.

The drug molecules chosen for the study is paracetamol and Cytochrome C. These were tried to be loaded using different mechanisms. Paracetamol showed no detectable loading via incorporation method while the breathing in mechanism showed considerable loading at high temperature (61% and 16 mg drug/mg polymer) and low pH (60% and 14 mg drug/mg polymer). In order to enhance the loading and encapsulation efficiencies, different paracetamol and polymer concentrations were tried out. The highest loading – and encapsulation efficiency have been achieved with high paracetamol concentration (66 mM), while increasing the polymer concentration increases loading efficiency and decreases the encapsulation efficiency. This indicates that the available sites for drug interaction are enhanced with higher concentration of polymers while more drugs can attach at the same polymer concentration owing to a higher drug concentration gradient (high paracetamol concentration). The high loading observed at high temperature and low pH is a proof of the hypothesized hydrophobic interactions between the paracetamol and the nanogels.

Successful release of paracetamol has been achieved by increasing the temperature of the loaded paracetamol solution to 70 °C. The corresponding release of paracetamol measured after an hour was found to be 95%. Further increase in temperature has been assumed to cause a squeezing release of the drug from the nanogels. The release could also be observed when increasing pH from 3 to 7 (46% release). Drug delivery applications require controlled release at or close to body temperature (~37 °C) or in a region of low pH (tumour pH ~4). Therefore, the paracetamol loaded nanogels show less promising applications towards the required goal. Hence, it was decided to study the loading and release kinetics of a hydrophilic protein – Cytochrome C as an example of a molecule that shows conformational changes with pH.

Cytochrome C (8 μM) has been successfully loaded to the nanogels (2 mg/mL) with high loading – and encapsulation efficiencies of 86% and 0.17 mg drug/mg polymer respectively. Unlike the standard protocol used for measuring free paracetamol concentration (centrifugation using filters); Cytochrome C was analyzed by measuring the bound protein (after the free Cytochrome had diffused out through the dialysis membrane).

Release studies for Cytochrome C have been done under three different conditions, while the temperature has been kept constant at 39 °C. A release of 11%, 22% and 24% (after 24 hours) of the loaded Cytochrome has been observed in PBS, at pH of the polymer-drug solution and at pH 3 respectively. Although, the release kinetics has been observed to be slow,
it is believed that a combination of high temperature and low pH can make the release faster and more efficient. The high retention capacities of the nanogels enable successive cycles of drug release over sustained periods. These nanogels can be further studied by modifying the surface using Poly(Ethylene glycol) (PEG) or/and binding to metallic NPs to introduce theranostic properties. Multiple drugs loading can be achieved with these highly functional nanoconstructs.
6 Future work

6.1 Drug release studies of Cytochrome C
Cytochrome C shows possible changes when heating it. That is why it would be interesting to look at the loading/release kinetics dependent on the pH only. Conformational changes can occur at different pH’s, which would make these very relevant studies of the protein.

Cytochrome C shows release at elevated temperature. This release rate is believed to increase if increasing the temperature and lower the pH. Since higher release rate is observed at pH 3, this pH would be interesting to use when releasing the drug at 45 °C. However, the temperature effect on the morphology of the protein should be mapped. In addition, to utilize this high temperature the surface of the hydrogel network needs to be modified.

The successful loading and the release of Cytochrome C shows that similar hydrophilic molecules like siRNA, pro-drugs and peptides can also be studied using these nanogels.

6.2 Polymers incorporated to magnetic NPs.
One modification of the polymers is to incorporate then with magnetic NPs. This is shown in Figure 51 with gold NP incorporated with functionalized polymers.[65]

![Figure 51 – Shows example of a metal NP incorporated with functionalized polymers.[65]](image)

The inorganic NPs are defined as one of the two nanocarrier categories which consist of inorganic and organic/polymeric nanocarriers. The inorganic nanocarriers, such as mesoporous silica, magnetic NPs, gold NPs and quantum dots, possess capabilities for tracking and their rigid surface can be functionalized. The organic/polymer nanocarriers will be highly flexible in terms of chemistry and structure. Along with the polymeric NPs (nanogels etc.) are the micelles, dendrimers and liposomes which consist of amphiphilic copolymers with biocompatibility.[6] The polymeric and inorganic NPs can be combined to give multiple functions. This is relevant when considering that the VPTT for PNIPAm/AAc nanogels is higher than the body temperature. To trigger the de-swelling, magnetic field can be used if the hydrogel is modified with metal NPs. This will lead to de-swelling transitions
and a squashing release of drugs when heated magnetically.[9] For example, PNIPAm/AAc coated Au - NPs show a decrease in size at high temperature and low pH.[19]

It would also be interesting to try to transport the drug carriers while over the VPTT, since the particle size is smaller and thereby they are more readily taken up within cells. Increased uptake above the transition temperature has been proven by Choi et al. by introducing sub-micrometer particles into cells in a temperature-dependent manner. They showed that molecularly design stimuli-sensitive hydrogels of PNIPAm/AAc could serve as useful carriers for intracellular delivery of macromolecular drugs.[8]

Another study has confirmed effective heating with Fe–containing PNIPAm microgels. These NPs were heated by a local oscillating magnetic field and de-swelling was observed with increasing temperature. This type of heating is less restricted than heating by light (restriction of human tissue), but a powerful enough magnetic source is required to achieve localized heating.[9]

A possible problem using metal NP is the shallow light penetration when heating. This needs to be further investigated, along with the investigation of non – ideal behavior of the gels, such as inhomogeneous distribution within the gels (shell formation), incomplete drug loading and slow/incomplete drug release. When these factors are mapped, they can perhaps be avoided, and the opportunities that come with gels as delivering systems can be fully utilized.[9]

### 6.3 Incorporation of PEG

The NPs can also be functionalized PEG (MW – 1000) to give longer circulation time in the blood, and thereby increase the possibility for the NPs to reach the desired target.[5, 16] This is given as an example in Figure 52, with a PEGylated NP which is used as drug delivery system.[66]
Drug carriers with a PEGylated interface have shown to make it difficult for body proteins to adhere during circulation of the nanocarriers inside the body. This is due to PEG’s hydrophilicity, high biocompatibility and high conformational flexibility.[16] This polymer can reduce the toxicity in vivo and enhanced permeation of the nanocarriers.[6] In addition, PEGylated proteins have shown to be able to evade premature clearance through RES. For example in delivering of diabetes type 2 drugs, the half-life was increased from two hours to more than 100 in the circulation time when incorporated with a PEG-like hydrophilic polypeptide.[5] Due to PEG’s positive charge (the cells are negatively charged), the polymer will also provide more readily transport into the cells.[8] Efficient internalization in endosomes and cytosol has for example been achieved by surface modification with PEG onto gold NPs.[4]

PEG incorporated to PNIPAm has shown reversibly temperature-dependent swelling/deswelling transitions. These systems have reduced solvency and increased de-swelling with increased temperature.[9] PEG also showed to change the phase transition temperature. Due to the incorporation of the hydrophilic polymer the particle requires more thermal energy to collapse. This has been proven with PNIPAm microgels cross-linked with BIS, which originally had a VPTT of 31°C increased to 36 °C. In addition the collapse happened over a wider temperature range.[16]

Over the volume transition of polymer network the polymer will have twice the level of protein adhesion compared to the swollen state, but by incorporation of PEG this adhesion can be significantly reduced, both over and under the volume transition.[16]

Charge modifications with PEG of the surface give the NPs the ability to alter the electrostatic BBB permeability more easily, which is very difficult to cross even today. This gives new hope for delivering drugs to difficult reachable sites over the BBB, like to brain tumors and the central nervous system (for example in treatment for Alzheimer’s, prion disease and many other diseases without cure today).[4]
7 Bibliography

40. Bandyopadhyay, S., Personal communication, 2014.
# Hazardous activity identification process

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**Unit:** (Institute)  
**Line manager:** Edd Blekkan  
**Participants in the identification process (incl. function):**  
Professor Wilhelm R. Glomn (supervisor), Marte K. Andersen (student)  
**Short description of the main activity/main process:**  
Master thesis in smart nanoparticles for targeted drug delivery  
**Is the project work purely theoretical? (YES/NO):** NO  
**Answer “YES” implies that supervisor is assured that no activities requiring risk assessment are involved in the work. If YES, skip rest of the form.**

**Signatures:**  
**Responsible supervisor:**  
**Student:**
Risk value = Likelihood (1, 2 ...) x consequence (A, B ...). Risk value A1 means very low risk. Risk value E5 means very large and serious risk.

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<td>Medium: Once a year or less</td>
<td>C</td>
<td>Dangerous</td>
<td>Serious personal injury</td>
<td>Minor damage. Long recovery time</td>
</tr>
<tr>
<td>4</td>
<td>High: Once a month or less</td>
<td>B</td>
<td>Relatively safe</td>
<td>Injury that requires medical treatment</td>
<td>Minor damage. Short recovery time</td>
</tr>
<tr>
<td>5</td>
<td>Very high: Once a week</td>
<td>A</td>
<td>Safe</td>
<td>Injury that requires first aid</td>
<td>Insignificant damage. Short recovery time</td>
</tr>
</tbody>
</table>

**Matrix for Risk Assessment**

<table>
<thead>
<tr>
<th>Consequence</th>
<th>Very critical</th>
<th>Critical</th>
<th>Dangerous</th>
<th>Relatively safe</th>
<th>Safe</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E1</td>
<td>E2</td>
<td>E3</td>
<td>E4</td>
<td>E5</td>
</tr>
<tr>
<td></td>
<td>D1</td>
<td>D2</td>
<td>D3</td>
<td>D4</td>
<td>D5</td>
</tr>
<tr>
<td></td>
<td>C1</td>
<td>C2</td>
<td>C3</td>
<td>C4</td>
<td>C5</td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>B2</td>
<td>B3</td>
<td>B4</td>
<td>B5</td>
</tr>
<tr>
<td></td>
<td>A1</td>
<td>A2</td>
<td>A3</td>
<td>A4</td>
<td>A5</td>
</tr>
</tbody>
</table>

**Explanation of the colors used in the risk matrix.**

<table>
<thead>
<tr>
<th>Color</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>Unacceptable risk. Safety measures must be implemented.</td>
</tr>
<tr>
<td>Yellow</td>
<td>Measures to reduce risk shall be considered.</td>
</tr>
<tr>
<td>Green</td>
<td>Acceptable risk.</td>
</tr>
</tbody>
</table>
Appendix B – Calculations

Paracetamol:

Calculation example of the loading efficiency when the pH is adjusted to 3 and stirred for 30 minutes is shown in Equation 1, 2 and 3.

\[
\text{Loading efficiency: } \frac{\text{Amount drug loaded}}{\text{Amount total drug added}} \cdot 100\% \tag{1}
\]

\[
\text{Loading efficiency: } \frac{C^0 - C^1}{C^0} \cdot 100\% \tag{2}
\]

Where \( C^0 \) is the conc. of paracetamol before loading to the nanogels and \( C^1 \) is the conc. of the filtrate (free drug) after the loading of paracetamol to the nanogels.

\[
\text{Loading efficiency: } \frac{\frac{mg}{ml} - \frac{mg}{ml}}{\frac{9.6mg}{ml}} \cdot 100\% = 59\% \tag{3}
\]

The calculation of the corresponding encapsulation efficiency is shown in Equation 4, 5 and 6.

\[
\text{Encapsulation efficiency: } \frac{\text{Amount drug loaded}}{\text{Amount polymer added}} \tag{4}
\]

\[
\text{Encapsulation efficiency: } \frac{C^0 - C^1}{C_p} \tag{5}
\]

Where \( C_p \) is the concentration of the polymer added to the drug solution.

\[
\text{Encapsulation efficiency: } \frac{\frac{mg}{ml} - \frac{mg}{ml}}{\frac{2mg}{ml}} = \frac{2.8 \text{ mg paracetamol}}{\text{mg polymer}} \tag{6}
\]

Release:

The calculation of release when heating the solution to 70 °C is shown in Equation 7.

\[
100\% - \text{loading efficiency} = 100\% - 5.1\% = 95\% \tag{7}
\]

The calculation of the release when the pH is increased to 7 from 3 is shown in Equation 8.

\[
\frac{C_{\text{Loaded at pH 3}} - C_{\text{Loaded at pH 7}}}{C^0} \cdot 100\% = \frac{\frac{C^0 \cdot \text{loading efficiency}}{100} - \frac{C^0 \cdot \text{loading efficiency}}{100}}{C^0} \cdot 100\% =
\]
\[
\left( 9.6 \frac{mg}{ml} \times 0.5882 \right) - \left( 9.6 \frac{mg}{ml} \times 0.1315 \right) = 46 \%
\]

(8)

**Cytochrome C:**

The calculation of the loading – and encapsulation efficiency when the drug is loaded at normal conditions (pH 4.4) after 24 h is shown in Equation 9, 10 and 11.

**Loading efficiency:**

\[
\frac{C^0 - (C^0 - C^1)}{C^0} \cdot 100 \% = 85 \%
\]

(9)

Where \( C^0 \) is the concentration of drug/polymer solution before loading and \( C^1 \) is the concentration after loading (the Cytochrome C left in the polymer solution).

\[
\frac{0.2 - (0.2 - 0.17)}{0.2} \cdot 100 \% = 85 \%
\]

(10)

**Encapsulation efficiency:**

\[
\frac{C^0 \cdot \text{loading efficiency}}{100 \cdot C_P} = \frac{0.2 \frac{mg}{ml} \times 0.853}{2 \frac{mg}{ml}} = 85 \mu g \text{ drug/mg polymer}
\]

(11)

Where \( C_P \) is the concentration of the polymer in the drug solution.

**Release:**

The calculation of the release at pH 3 and after 1 hour is shown in Equation 12.

\[
\frac{\text{Amount released}}{\text{Amount loaded}} \cdot 100 \% = \frac{C^0 - C^1}{C^0} \cdot 100 \% = \frac{C^0 - (C^0 \cdot \text{loading efficiency})}{C^0 \cdot \text{loading efficiency}} \cdot 100 \%
\]

\[
= \frac{0.1204 \frac{mg}{ml} - (0.1204 \times 0.836)}{0.1204 \times 0.836} \cdot 100 \% = 20 \%
\]

(12)
Appendix C – Calculations of the VPTT
The VPTT for the hydrogels marked in unfilled circles shown in Section 4.3.4 is calculated with Equation 13 (from the first three points) and 14 (from the five last points).

\[ y = -4.68x + 429.1 \]  
(13)

\[ y = -22.69x + 1077.4 \]  
(14)

These two equations were put equal to each other and the VPTT was calculated as shown in Equation 15 and 16.

\[ y = -4.68x + 429.1 = -22.69x + 1077.4 \]  
(15)

\[ x = \frac{1077.4 - 429.1}{22.69 - 4.68} = 36 \, ^\circ C \]  
(16)