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Degradation and Stability of PBCA and POCA Nanoparticles

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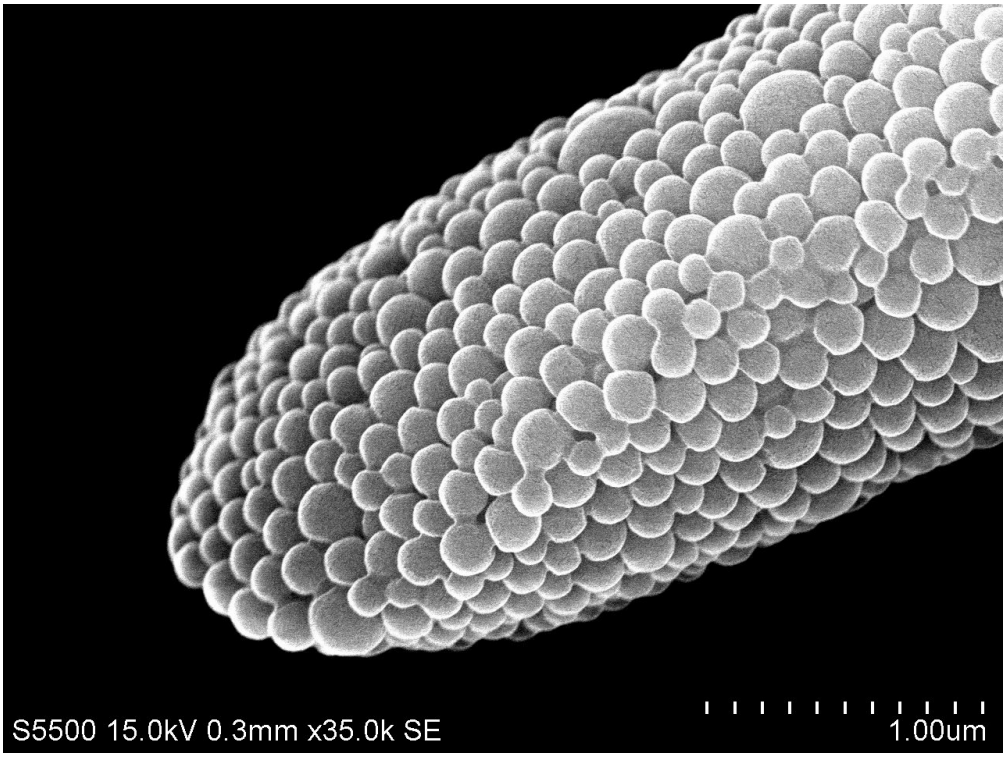

Freeze dried PBCA nanoparticles. Image reproduced from Bøe [4]

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Preface

This project is carried out mainly at the NTNU Nanolab through the Department of Physics at the Norwegian University of Science and Technology and at the laboratoy of SINTEF Materials and Chemistry. I would like to give a special thank to several persons that have helped me during this project, but especially to my supervisors Yrr Mørch and Catharina de Lange Davies. Yrr Mørch has given me personal guidance to the laboratory work and to the progress of the project. Catharina de Lange Davies has given me personal advice and guidance on the results and in the writing process of this paper. I would also give a thank to the staff at SINTEF Materials and Chemistry that has produced the particles involved in the project and given me sufficient training to run the different instruments there. Additionally they have been very helpful and friendly, and it has been a very constructive environment to work in. Analysis were also performed on Gas Chromatography by guidance from senior Engineer Susana Villa Gonzales from the Department of Chemistry.

Sammendrag

Nanopartikler er lovende kandidater for bærere av medisin for kontrollert medisinlevering. I dette prosjektet er tre ulike instrumenter (Zetasizer, Nanosight og gasskromatografi) brukt til å detektere og analysere nedbrytning av monodisperse poly butylcyanoacrylate (PBCA) og poly octylcyanoacrylate (POCA) nanopartikler med en gjennnomsnittlig størrelse på henholdsvis 145 og 155 nm. Nanosight og gasskromatografi viste seg å være verdifulle instrumenter for å måle nedbrytning, mens Zetasizer-en ga uproduserbare resultater på grunn av for høy polydispersitet i prøvene. PBCA og POCA partiklene ble tested gjennom to ulike nedbrytningsoppsett. Det ene innebar et dialyseoppsett i romtemperatur, hvor mediumet kontinuerlig ble fornyet. Det andre oppsettet var bruk av reagensflasker plassert i en ovn ved 37°C. I dialysemetoden ble det testet hva slags påvirkning ulike pH-er (4.0, 5.5 og 7.4) hadde på nedbrytningen av partiklene. I reagensflaskene ble ulike mediers påvirkning testet, deriblant cellemedium, blodserum og buffer pH 7.4 med og uten enzymet esterase.

Ut ifra disse eksperimentene ble det klart at PBCA partikler brytes signifikant raskere ned enn POCA partikler i alle testede mediumer. I tillegg viste deg seg at PBCA partiklene ble svært forskjellig påvirket av de ulike pH-ene. Ved pH 4.0 var det lite (10%) eller ingen nedbrytning. Ved pH 5.5 var omtrent 70% av alle partiklene brutt ned etter 30 dager. Ved pH 7.4 avtok konsentrasjonen av partikler proporsjonalt med en $\frac{1}{x}$-funksjon, hvorav 37% av partiklene var brutt ned etter bare 3.5 timer. Nedbrytning av POCA partikler derimot, viste ingen tegn til pH-avhengighet, og oppførte seg likt ved all pH-ene.

Nedbrytningshastigheten i blodserum var omtrent tilsvarende som i buffer pH 7.4, mens i cellemedium var den litt langsommere. I tillegg viste det seg at den gjennomsnittlige partikkelstørrelsen i cellemedium og blodserum var betydelig større, noe som skyldes adsorpsjon av proteiner på overflaten.

Abstract

Nanoparticles represent promising carriers for controlled drug delivery. In this project three different intruments: Zetasizer, Nanosight and Gas Chromatography, have been used to detect and analyse degradation of monodisperse poly butylcyanoacrylate (PBCA) and poly octylcyanoacrylate (POCA) nanoparticles with a mean size diameter of 145 and 155 nm respectively. It was found that the Nanosight and Gas Chromatography are valuable instruments for detecting and analysing degradation, whereas the Zetasizer turned out to give unreliable results because of increasing polydispersity in the samples. PBCA and POCA particles were tested in two different setups. One including a dialysis setup in room temperature, in which the solvent was regularly exchanged. The other consisted of reagent bottles held in an oven at 37°C. In the dialysis method the influence of buffers with pH 4.0, pH 5.5 and pH 7.4 were tested. In the reagent bottles different mediums were tested, like cell medium, blood serum and buffer pH 7.4 with and without the enzyme esterase.

From these experiments it became clear that PBCA particles degraded significantly faster than POCA particles in all tested mediums. Degradation of PBCA particles were also strongly affected by the pH. At pH 4.0 there was little (10%) or no degradation still after 30 days. At pH 5.5 there was significant more with 70% degradation after 30 days. At pH 7.4 the concentration decayed proportionally to a $\frac{1}{x}$-function, in which 37% of particles in buffer pH 7.4 have been degraded after just 3.5 hours. POCA, on the other hand, showed no sign of pH-dependent degradation, and the particles behaved similiarly in all the three pHs.

The degradation-rate for PBCA and POCA in blood serum was approximately similar as in buffer pH 7.4, whereas in cell medium it was slightly slower. Additionally, the mean size increased distinctly in cell medium and blood serum due to protein adsorption.

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Chapter 1

Introduction

1.1 Cancer - a major worldwide problem

Cancer is one of the most serious health problems in the world today. Each year it is responsible for around 13% of all human deaths (7.6 millions in 2008).[48] It strikes gender of both kind, young and old and people from all over the world. Cancer is a very complex problem, and cannot be classified as one disease, rather like 200 different ones with some common characteristics. The treatment of cancer is continuously getting better, and from some cancer types most patients survive. However, for other types the conventional treatments like, surgery, radiation theraphy and chemotheraphy, are not good enough. The main limitation of these methods is the lack of selectivity, and the bad side effects that occur because of this.

The state of the art within cancer research is the development of a drug delivery system, in which this main limitation is supposed to be overcome. With this method the drug is delivered effective and in high doses only to diseased cells, whereas healthy ones are left unharmed behind. This would lead to a higher survival rate, decreased treatment periods and quicker recoveries. The first products are already on the market,[1, 17] but many are still to come. In the coming decades the cancer treatment will positively be revolutionized.

1.1.1 Purpose of project

In this project, carried out by SINTEF/NTNU, it is used a self-made drug carrier[1], in which drug is loaded into polymeric nanoparticles. One important part of this project has been to find a good way to measure and quantify degradation of polyalkylcyanoacrylate nanoparticles. Degradation and drug delivery is often connected, and therefore only by understanding the mechanisms of degradation and which parameters that are affecting it, it is possible to make an effective drug delivery system. Vaksdal [66] tried two years ago to measure degradation based on dry-weight measurements before and after degradation, but this turned out to be very difficult. The concentration was too high, the particles too unstable and this lead to particle aggregation. Secondly it was hard to quantify the accurate

[1]The particles are produced by SINTEF Materials & Chemistry

mass of the particles. Last year Bøe [4] saw by electron microscope that the particles of Vaksdal were aggregated and only surface particles had been degraded. The bulk particles remained undegraded. Additionially, Bøe found that nanoparticles could degrade even in water if the concentration was low enough ($\leq 1:250$). This knowledge will be be used as a basis for further degradation test in this project.

Chapter 2

Theory

2.1 What is a drug delivery system?

A drug delivery system is basically a method where the drug is encapsulated in some kind of drug carrier. The drug carrier is simply a designed capsule to transport the drug safely to the desired end-station. This carrier goes ideally 'unseen' through the blood and will finally reach its final destination, which happens to be the tumor. This can happen either through unspecific accumulation, in which the drug carrier accumulate in tumor tissue due to leaky blood vessels (see section 2.6), or through target-seekening drug carriers which have a special molecule attached onto the surface which binds to a specific receptor that is overrepresented in cancer tissues. When the drug carrier is located at the right position it will somehow be opened or degraded to release the drug. It can happen either by an external stimulus, which could be a laser, an ultrasound field, a magnetic field, or through the internal chemical environment, for example like chemical degradation by enzymes. The drug will then be taken up by the surrounding cells, which now happen to be cancer cells.

2.2 Drug Carriers

A drug carrier is some kind of capsule or particle that is designed to carry a load, for example a drug or a hormone, to some particular destination. The size of the capsule can vary but is usually in the submicron range. Certain criterias are common for all drug carrier and must be met in order to be accepted as a drug carrier. First of all, it must be biocompatible, which means no negative reactions when placed in the body. Ideally it should go unnoticed through the body. It needs to be biodegradeable, or at least excretable by the kidneys to avoid an accumulation in the body, which again can lead to a toxic reaction. The circulation time of the drug carrier must be sufficently long in order to bring the drug carrier load to its right destination. Finally, the drug carrier should somehow be selective, in the way that it accumulates only in the diseased regions, and leave healthy cells unharmed behind. The concentration of the particles in target region should be high enough that an effective treatment is possible. The design of the capsule is often quite complex in order to fulfill all

these criterias. Different capsules designs and materials are used for different tasks. The most common drug carriers are micelles, liposoms and polymeric nanoparticles. [19, 24, 31]

2.2.1 Micelles

A micelle (Figure 2.1) is build up of amphiphilic molecules, which means that they have a lipohilic(fat-loving) tail and a hydrophilic(water-loving) head. Such molecules are solube in water, but only for small concentrations. In water, the lipophilic part will be surrounded by watermolecules which arrange themself in a cage-like structure. This, however, is a very energy-demanding system, and since a system always try to minimze its energy, this is not an ideal situation. When the concentration increases above the critical micelle concentration (CMC) the system becomes so unfavourable that the configuration changes spontaneously. For concentrations above the CMC, amphiphilic molecules arrange themself into a micelle, which is a spherical object. In the case of a water as the solvent, the hydrophilic parts are pointed outwards, whereas the lipophilic parts are hidden inside the core of the micelle.

Hydrophobic drugs can be sequestered in the oily core of the micelle, but because of its size (5-30nm) the loading capacity is very limited. Micelles are small enough to avoid the Mononuclear phagocyte system (MPS)(see section 2.5.3), and will quickly extravasate through interstital tissue. However, since the stability of the micelles are concentration dependent (>CMC), it is a possibility that they will break up when distributed in the body, and the load will be delivered before they have reached their target destination. [23, 50]

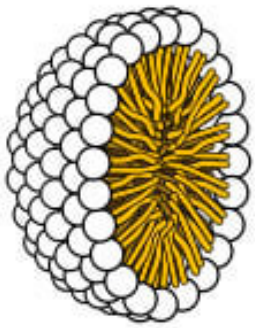


Figure 2.1: An illustration of a micelle. The hydrophilic head is pointing outwards, whereas the lipophilic tail is pointing inwards. Image reproduced from `chemistry.about.com`

2.2.2 Liposomes

A liposome (Figure 2.2) is another structure that can be build from amphiphilc molecules. Here, the hydrophilic head has just a slightly larger cross-section than the lipophilic tail, which makes the micelle-structure energetically unfavourable. In the case of the liposome, a spherical bilayer forms instead, and it is almost like a small cell, just without functional parts. A typical size of a liposome is much larger than for a micelle, and range from 80-200

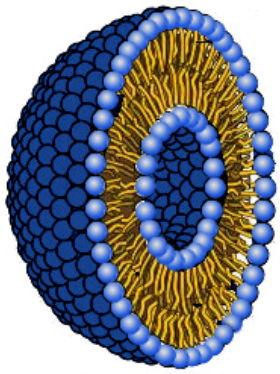


Figure 2.2: An illustration of a liposome. Image reproduced from `www.supplementclinic.com`

nm. A liposome is used for several drug-delivery purposes and can carry hydrophilic drugs in the core and hydrophobic drugs in the bilayer. Liposomes are for instance used for DNA transfection into a cell, a technique known as *lipofection*. It should be pointed out that the formation of liposomes is not a spontaneous process. Energy must be added to the system, for example by sonification, to make liposomes. In contrast to micelles, liposomes are cleared by the MPS, but by addding PEG (see section 2.5.3) on the surface, the liposomes can be made 'invisible' for the MPS-macrophages and the circulation time can be extended. [23, 50, 65]

2.2.3 Polymeric nanoparticles

The third common drug carrier-type, which is becoming more and more popular, is the polymeric nanoparticles (Figure 2.3). There are two main types: the nanosphere and the nanocapsule. The nanospheres are matrix systems constituted by the polymer and the drug is physically and homogeneously dispersed. The nanocapsules are vesicular systems, in which the drug is solubilized in a liquid core, either water or oil, surrounded by a thin polymer layer. The size of these can range from a few tenths of nanometer to several hundreds, and they are often made in a miniemulsion technique (see section 2.3.2). A range of different polymers can be used, but the most common ones are biodegradeable polyesters, poly alkyl-cyanoacrylates or natural polymers. The greatest advantage of polymeric nanoparticles are their high payload. When the name nanoparticle is mentioned later in this text, the meaning is always a nanosphere, because this project is carried out only with nanospheres. [25, 68]

2.2.4 Drug carriers approved for clincal trials

Even though research on this field has been carried out for several decades, the number of products in the clinical is still relatively small. Abraxane [1], which is an albumin-bound form of paclitaxel, and Doxil [17], which is a liposome based particle, are two of the products that have passed the approval of FDA (U.S Food & Drug Administration) and show very

promising results on pasients. However, several products are up and coming, and with the intensive research going on more and more products are expected to get the approval.

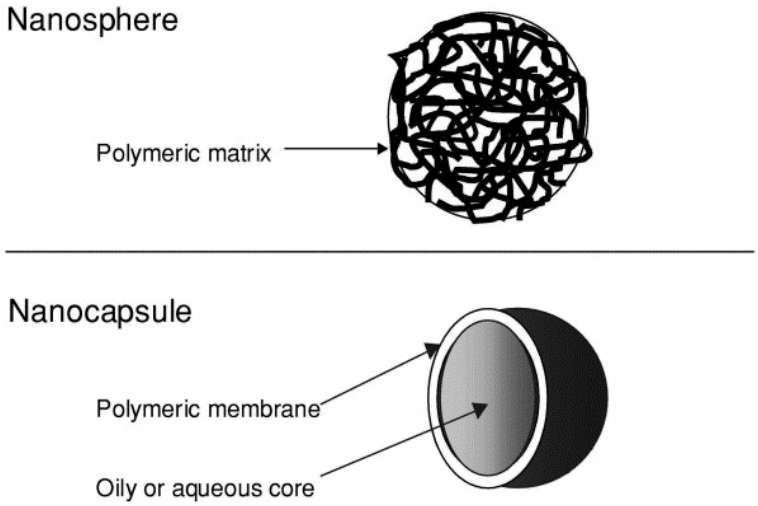


Figure 2.3: A polymeric nanosphere and a polymeric nanocapsule. Image reproduced from `www.sciencedirect.com`

2.3 Poly alkylcyanoacrylate nanoparticles

The nanoparticle used in this project are made of a poly alkylcyanoacrylate (PACA), namely poly butylcyanoacrylate (PBCA) and poly octylcyanoacrylate (POCA). PACA was first introduced into the market as surgical glue. [69] Now, it is in focus because of its promising results in drug delivery systems. There are many different types of PACA nanoparticles (Figure 2.4). The only molecular difference between them is the alkylchain. The alkyl chain, however, affect the rate of degradation, and the release rate of the degradation products is found to be correlated with the toxicity.[27, 37] Today, PBCA is the most common one, but POCA is getting more attention because of its low toxicity.[27] They have an average size around $100 - 300\,\text{nm}$, are sufficiently biocompatible [80] and show a stability over months when held in a high concentrated solution at pH 4. [56] The biocompatibility and stability depends on parameters like size, degree of polymerisation, PEG-length and -density.

2.3.1 Polymerisation of alkylcyanoacrylates

Alkylcyanoacrylate-monomers are highly reactive compounds and in their pure form they are very difficult to handle.[36, 53, 69] They show a remarkable tendency to polymerize and inhibitors are therefore essential to maintain their stability. The polymerisation of alkylcyanoacrylates can theoretically happen through three different pathways, namely free radical, anionic or zwitterionic polymerisation (Figure 2.5). Anionic and zwitterionic routes are favoured because they are easier to control than radical polymerisation at conventional

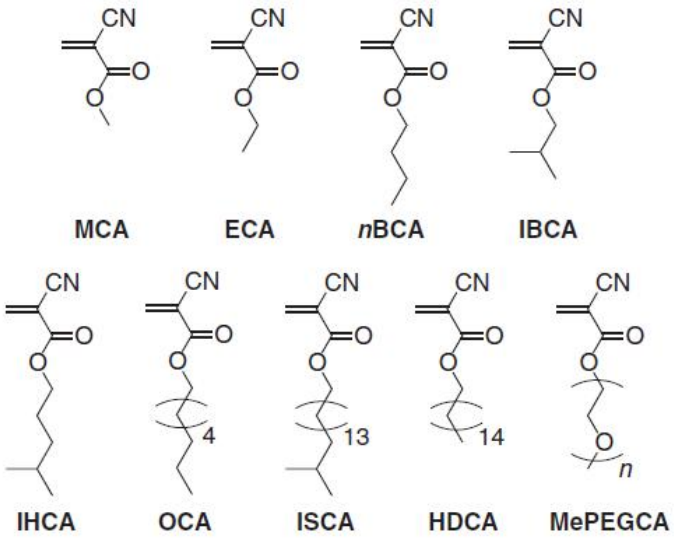


Figure 2.4: Structure of the alkyl cyanoacrylates described in the literature: methyl cyanoacrylate (MCA), ethyl cyanoacrylate (ECA), n-butyl cyanoacrylate(nBCA or just BCA), isobutyl cyanoacryalte (IBCA), isohexyl cyanoacrylate (IHCA), octyl cyanoacrylate (OCA), isostearyl cyanoacrylate (ISCA), hexadecyl cyanoacrylate(HDCA), and methoxypoly(ethylene glycol) cyanoacrylate (MePEGCA). Image reproduced from Nicolas and Couvreur [46].

experimental condition. Most of the studies are therefore performed by anionic and zwitterionic polymerisation.[46, 69] The most common initiators of anionic polymerisation are anions (like I^-, CH_3COO^-, Br^- and OH^-), weak bases (like alcohols), water and amino acid found in living tissue, with the common denominator that they have a nucleophilic group.

2.3.2 Miniemulsion - synthesis of the particles

Miniemulsion polymerisation was first carried out by Couvreur et al. [11] and is one of the most common approaches used to synthesize filled polymeric nanoparticles (nanospheres). The polymerisation is initiated by the hydroxyl ions of water. Anionic polymerisation can be controlled in aqueous medium because of it is highly dependency on the pH, and hydrochloric acid can therefore be used to control the polymerisation.

In the miniemulsion process (Figure 2.6) of making the PACA nanoparticles the first step consist of mixing a surfactant and two immiscible liquids, in which one of them is the monomer. They will form an emulsion, in which the monomer is dispersed as droplets in a continuous medium stabilized by the surfactant. It has been found that the nature and concentration of the surfactant play an important role on the particle size, whereas the type of monomer and the surfactant have a great influence on the final molar mass of the polymer. In addition, the pH and the inhibitor concentration will also affect the nanoparticle

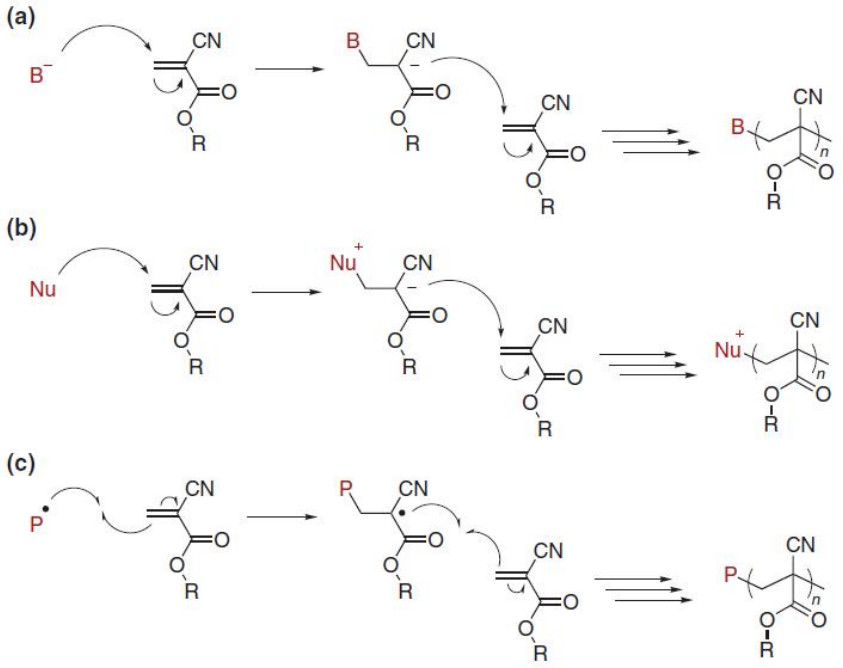


Figure 2.5: Initiation and propagation steps involved during (a) anionic, (b) zwitterionic, and (c) radical polymierisation of alkyl cyanoacrylate monomers initiated by a base (B^-), a nucleophile (Nu), and a radical ($P^\bullet$), respectively. Image reproduced from Nicolas and Couvreur [46].

properties. [14, 15] In the synthesis of PBCA nanoparticles butylcyanoacrylate (BCA) and hydrochlorid acid (HCl) are the two immiscible liquids. For POCA-particles, BCA is replaced by the monomer octylcyanoacrylate (OCA).

Different surfactants are available. The most common one to use is sodium dodecylsulfate(SDS), but Tween 80, Jeffamine and dioctyl sodium sulfosuccinate (DSS/AOT) are also used (Figure 2.7). Since strong acids are known to inhibit the polymerisation of BCA, it is used as the continuous medium. [46]

Ultrasonication is then used, in which shear forces split larger droplets into smaller ones. After a few minutes the size will reach an equilibrium based on parameters like the pH of the continuous medium, the concentration of the surfactants and the sonication time . However, after a characteristic sonication time, the droplet size cannot be reduced further.[74] As mentioned BCA is a very reactive monomer and a small heat change can be enough to trigger the polymerisation. The mixture is therefore held in an ice bath and the sonification is run in a pulse mode, to avoid uncontrolled spontaneous polymerisation.

The second step is the polymerization process, and is intiated by the adding of an initiatior. As mentioned in section 2.3.1, many different initiators are available, with the common denominator that they all have a nucleophilic group. For instance if the particles should have a PEG-layer, this component is now added to the solution and will act as an initiator. [78]

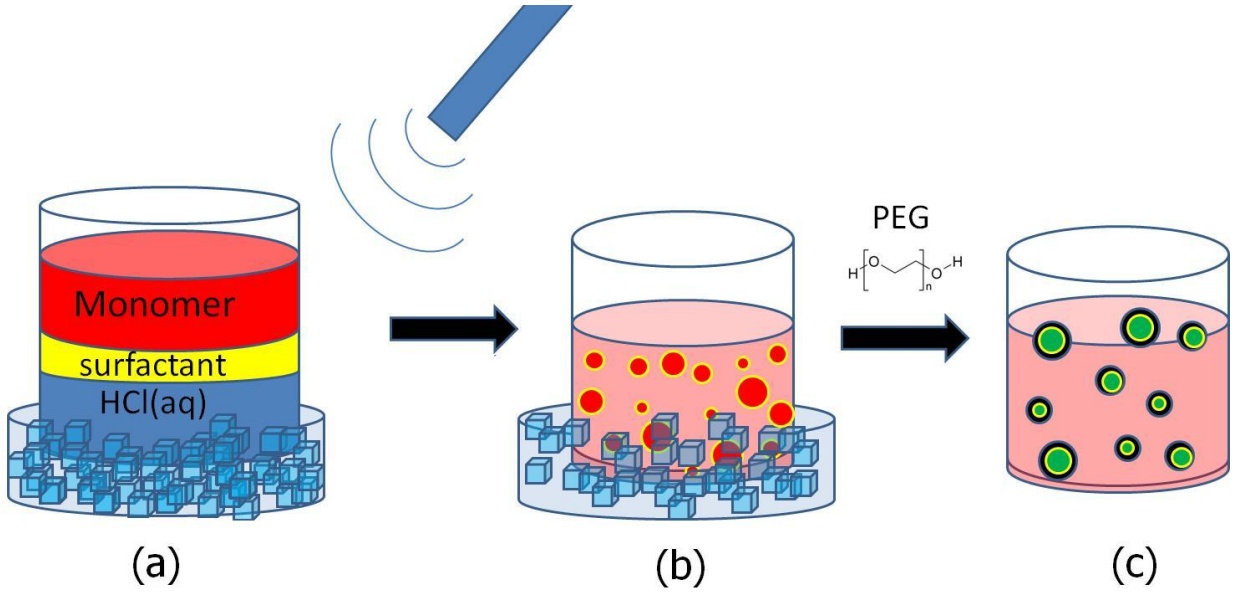
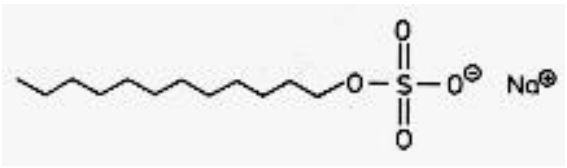
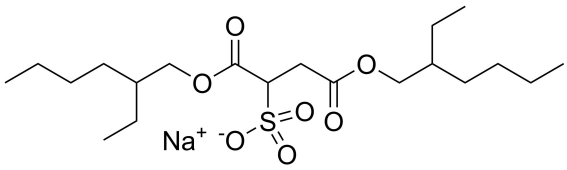


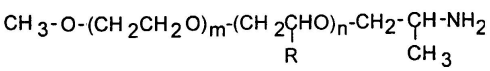
Figure 2.6: A schematic illustration of a miniemulsion process. In (a) the monomer is mixed with a surfactant and an acid medium. The mixture is by ultrasonification transferred to a miniemulsion (b), in which the monomer form tiny droplets stabilized by the surfactants. The ice bath is present to avoid spontanoeus polymerisation. (c) PEG is then added, which also initiate the polymerisation. Each droplet can now be regarded as a nanoreactor and each monomer droplet results in a polymeric nanoparticle.



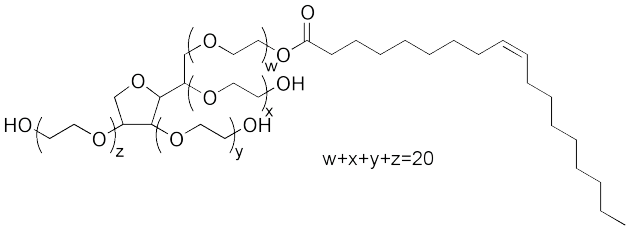
(a) SDS - Sodium dodecylsulfat



(b) AOT - Dioctyl sodium sulfosuccinate



(c) Jeffamine



(d) Tween 80

Figure 2.7: Different surfactants used in the synthesis of PBCA nanoparticles

The surfactants are stabilizing the droplets so effectively, that the polymerisation process is restricted to each monomer droplet. Each droplet can therefore be regarded as a nanoreactor, in which the droplets are transformed to nanoparticles in a one-to-one-ratio. [26] Destabilizing processes like Ostwald ripening[1] and coalescence are inhibited by the effective surfactant and the polymerization can take place without affecting the stability of the dispersion.

Even though this process seems to be straight forward, the polymerisation in acidic medium is not that trivial and proceeds via a stepwise anionic mechanism involving reversible propagation and reversible termination steps (Figure 2.8). PACA oligomers are formed in the monomer droplets and acid-inhibiting agent present in the monomer will reversibly terminate the process. Then still living chains will re-initiate the polymer process leading to further polymerisation until a molecular weight equilibrium is reached. [6]

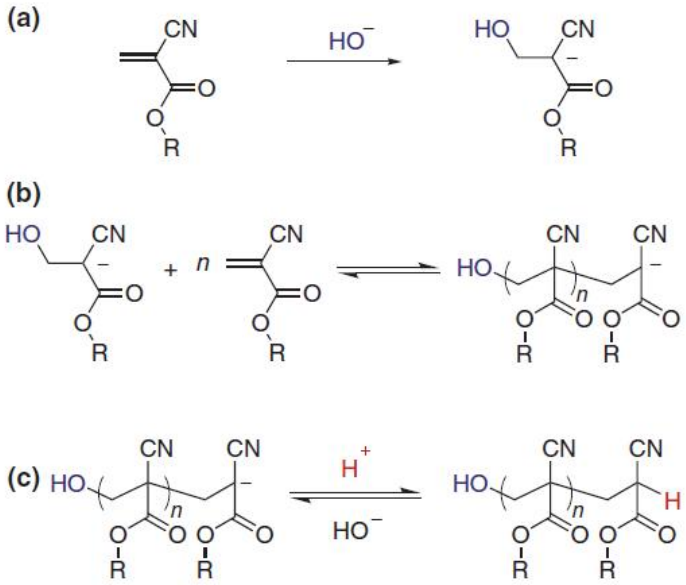


Figure 2.8: A schematic representation of poly(alkyl cyanoacrylate) formation via the stepwise anionic polymerisation mechanism in miniemulsion. Initiation step (a), reversible propagation step (b), and reversible termination step (c). Image reproduced from Nicolas and Couvreur [46].

Many drugs can be sequestered in PACA nanospheres, and some of them are reported to initiate the polymerisation reaction, which may lead to loss of their biological activity. [22] However, such covalent bindings may be favourable for systems where extra long stability is required. A series of photosensitizers which are used in phototheraphy in tumors are succesfully associated with nanospheres using this technique. [33]

The third step is to remove the surfactants and some uses this step also to increase the particle concentration. Especially SDS is important to remove because of toxicity concerns [12].

[1] Ostwald ripening is the process where large bubbles increase in size at the expense of smaller ones

The concentration is increased for better storage-conditions and clinical requirements.[69] A dialysis process is used for this process. Free surfactants are small enough to pass through the semipermeable membrane and will be driven by diffusion. Particles, on the other hand, are too big to pass through the membrane and will therefore stay in the dialysis bag. Osmosis is the spontaneous net movement of solvent molecules (water) through a partially permeable membrane into a region of higher solute concentration, in the direction that tends to equalize the solute concentrations on the two sides.[77] A more concentrated solution can therefore be otbtained when the surrounding medium is more concentrated than the nanoparticle solution. [68] Although almost all free SDS is completely removed, some SDS can still be adsorbed onto the particles. By now, one does not know whether or not this small amount has a negative effect or not. Anyway, for the future, ways to obtain non-toxic particles must be further investigated, either through better knowledge of SDS-toxicity, better removal of SDS or by using a new non-toxic surfactant in the synthesis process.

Targeting particles

In the synthesis it is also possible to implement targeting. The drug carrier will then have a special molecule attached on the surface. This molecule will bind to a receptor, which often is overrepresented or unique in the tumor-environment. An example of a targeting molecule is the aminoacid-sequence *arginine-glycine-aspartic acid* (RGD) which binds specific to the $\alpha_V\beta_3$-integrin, which is expressed on angiogenic endothelial cells, but not on resting endothelial cells.[64] This makes RGD to a very promising marker for tumor-vasculature.

2.4 Degradation of PBCA nanoparticles

The degradation mechanisms for PACA has been intensively studied, and theoretically there are several ways of degradation (Figure 2.9).

Leonard et al. [36] were the first to suggest a degradation pathway for PACA, namely the inverse Knoevenangel reaction. This pathway consists of hydrolysis of the polymer chain and results in the formation of butylcyanoacrylate and formaldehyde. This path of degradation will break the backbone of the polymer chain.

The second possible degradation pathway were proposed by Wade and Leonard [71] and involves ester-hydrolysis. A nucleophile (water or hydroxyl ion) initiate the reaction by attacking the carbon of the ester-group, which results in the water-soluble poly(2-cyanoacrylic acid) and a primary alcohol (butanol for PBCA). This was later confirmed by Leonard et al. [36]. These biproducts are water-soluble and can be eliminated in vivo by kidney filtration.[46] Esterase is a hydrolase enzyme that makes esters to split into an acid and an alcohol, and is found to catalyse the degradation process of PBCA.(See more in section 2.4.5)

The third degradation pathway, suggested by Ryan and McCann [53], is an unzipping depolymerisation reaction of the polymer chain, with an immediately repolymerisation resulting in lower molecule-weight polymer. The details of this pathway is still to be found, because of the difficulties of observing the mechanisms in the short time-interval of the

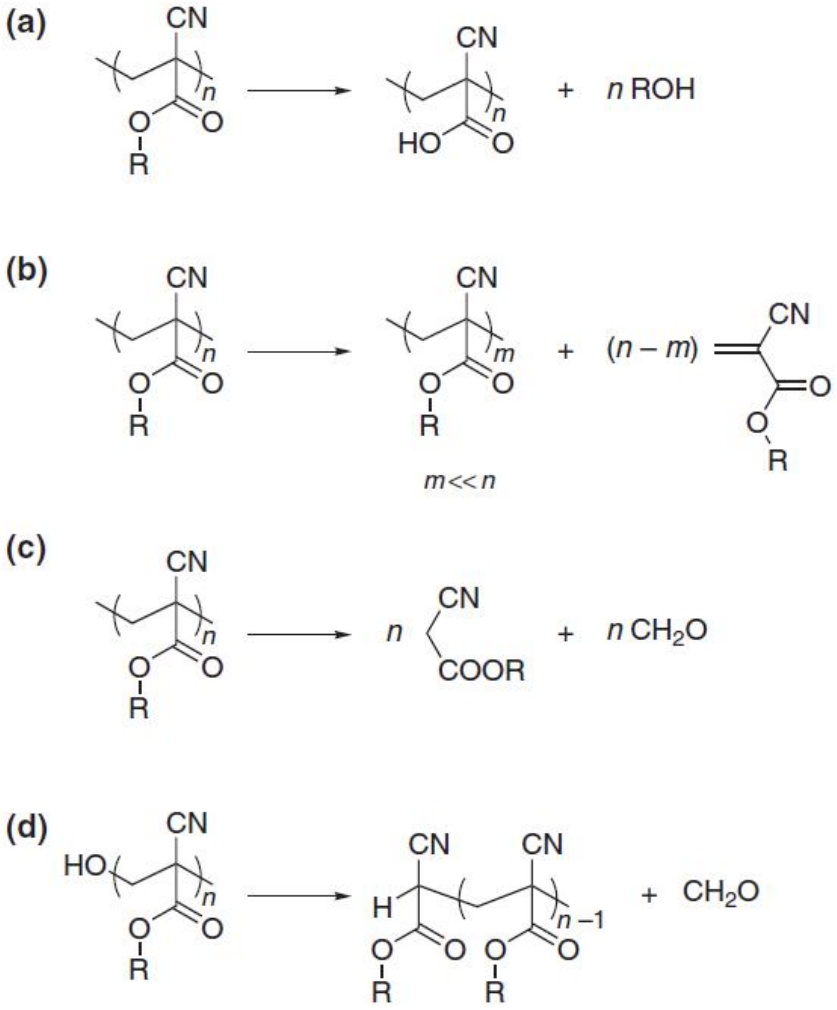


Figure 2.9: Possible degradation pathways for poly(alkylcyanoacrylate). (a) hydrolysis of ester functions resulting in poly(cyanoacrylic acid) and an alkylalcohol, (b) 'unzipping' depolymerisation reaction followed by repolymerisation, (c) inverse Knoevenagel reaction resulting in alkylcyanoacrylate and formaldehyde, (d) formaldehyde can also be produced by hydrolysis of the α-hydroxyl function of the polymer chains.

reaction.

All of these pathways are theoretically possible, but they will compete with each other. The inverse Knoevenangel reaction for example has been reported to a lesser extent in aqueous medium at physiological pH.[59] It will be too slow to compete with the enzyme-catalyzed ester-hydrolysis mechanism, which is found to be the main degradation pathway in biological environment.[35] In what degree the unzipping depolymerisation will occur, is unknown. However, in biological medium there is absolutely a possibility that it will occur.[53] The mechanism can namely be induced by amino acids of the proteins.

2.4.1 Surface degradation

It is proposed that degradation occurs at chain ends, and observed that the particles eroded and the size of the nanoparticles decreased with time.[70] Additionally, it is found that for PACA within a narrow molecular weight range ($10^3 < M_n < 10^4$) in buffer solution at pH 7.88 the degradation rate, k, is approximately proportional to $\frac{1}{M_n}$. Which means a direct relation to the polymer chain groups at the surface, since the polymer surface frequency of chain ends is inversely related to M_n, which thus determines k. [70]

A decrease of the particle size is frequently reported and is caused by surface erosion.[41, 61, 70] Theoretically there is also a chance that the particles will increase in size, either through flocculation [41] or because of swelling.

2.4.2 Swelling during degradation

Ion concentration differences across a semipermeable membrane causes an osmotic pressure gradient. Swelling is a phenomenon that is well known for polymer degradation.[34, 81] In a polymer system the difference in osmotic pressure results in an expansive force that swells the system until the force is balanced by the elastic force of the polymer network. This type of swelling is a mechanism that mainly occur for bulk degradation systems [34], in which water uptake by the system is much faster than polymer degradation. For surface eroding systems the degradation happens faster than water intrusion, and swelling is normally not a dominant mechanism.[55] However, theoretically there is great chance that swelling can occur for PACA nanoparticles. An interesting detail is namely that PACA during the degradation is stepwise transformed to a poly(cyanoacrylic acid), which is water soluble. In water at a neutral pH many of the acrylic acid side groups will loose their protons and get a negative charge ($R - COOH \rightleftharpoons R - COO^- + H^+$). The polymer becomes now a polyelectrolyte which leads to a change in the osmotic equilibrium.

Since a member of the polyacrylic acid familiy is created in the particles, the charges produced are not able to freely diffuse away from the particle. The particle can therefore be regarded as a semipermeable membrane in which water can flow in. In addition, the polymer will change from a relatively non-charged hydrophobic polymer to a highly charged hydrophilic polymer, in which polymer chains repel each other, and water can fill the space. Stretching of a polymer chain is energy unfavourable and the system will sooner or later come

to an equilibrium-condtion.[23] The polyacrylic acid is used in the industry as a superabsorber where this phenomenon is utilized. The polymer can then absorb water more than 1000 times its own weight.[38]

This type of swelling will depend on the salt concentration of the surrounding medium. The polymer will for instance swell less in a salt water solution than in pure water, because osmosis, the driving force, will then be limited. This type of swelling is not often reported for PACA particles, but at least once.[16]. However, for poly(lactic-co-glycolic acid) (PLGA) nanoparticles, it is reported more often.[30, 81] The swelling mechanism for PLGA is partly similar for the PACA, in which formation of carboxylic and hydroxyl groups changes the osmotic equilibrium. Others use controlled swelling to release the drug (see section 2.4.6).[60]

2.4.3 Degradation-dependence on pH

The pH has proven to have an affect on degradation rates for PACA particles. All studies report a faster degradation with increased pH.[35, 59, 70] Hydroxyl- ions (OH^-) are better nucleophils than water and a high pH will therefore give a faster degradation. However, as the hydrolysis goes on acid will be produced. If the solution is not buffered the hydrolysis will at one time stop, because the solution becomes to acidic. This will, however, not be a problem in the body, because the pH is very stable. The degradation rate is further enhanced by elevated temperature.[59] Long-term studies indicate that nanoparticles seem to be unchanged over several months if they are stored in very concentrated solution in acidic medium.[57] However, in basic envirionment the degradation time could be as fast as 24 hours for pH 10 at 50°C.[59] However, such results are very particle and concentration dependent, and such high pHs are not found in the body and are therefore not that relevant.

2.4.4 Degradation rate upon alkyl-length

The alkyl-length of the polymer has a great impact on the degradation rate. The hydrolytic degradation of alkyl-cyanoacrylates polymers increases with decreasing alkyl-chain length. An interesting point, is the fact that toxicity also seems to correlate with the alkyl-length. The toxicity seems to decrease with longer alkyl-length.[27, 41]

Huang and Lee [27] experimented with a copolymer made of poly butylcyanoacrylate and poly octylcyanoacrylate. They were able to control the degradation rate to a certain degree, and found that the hydrophobicity of the nanoparticles increased by increasing octyl-cyanoacryolate content, which makes it easier to incorporate hydrophobic drugs. However, the toxicity for the copolymer did not change.

Westrøm and Snipstad, two students working on the same project, found that the POCA showed no or little toxicity compared to the PBCA nanoparticles.[75] It is important to design a particle that fulfill both appropriate degradation-rate and acceptable toxicity, and by changing the alkyl-length or by making a copolymer these parameters may be tuned.

2.4.5 Degradation-dependence on enzymes

Degradation of PBCA nanoparticles in dog serum has been carried out [59], and almost complete hydrolysis was observed after 3.5 hours. If degradation in serum had occured only because of pH-dependent hydrolysis,the expected degradation would have been much slower, based on observed degradation in buffer. Thus, an enzymatic degradation mechanism was suggested to take place in serum.

The influence of different enzymes, namely amylase, pepsin and esterase, and their influence on degradation was further tested. [54] Esterase, which is a hydrolase enzyme that makes esters to split into an acid and an alcohol, was the only enzyme that had a distinct effect. Different types of it is found among others in blood, lysosomes, liver and kidney. [28, 52] , and esterase catalysed degradation is believed to occur as the major degradation pathway in vivo.

A biphasic enzymatic degradation was later reported [61], with the use of gas-chromatography. The butanol concentration was quantified and a burst release was present the first half hour, followed by a more gradual increase in butanol concentration the next hours. The high early concentration is presumably from the cleavage of ester side groups on the surface of the nanoparticles, and the gradual release(second phase) is possibly coming from hydrolysis of the polymer deeper into the particle. The second phase will be diffusionally hindered, because it is unlikely that enzyme molecules are able to penetrate the nanoparticle. Several studies have been performed to find how esterase influences the degradation. These studies do not always coincide with each other, and it is important to highlight that this behaviour could be different from particle type to particle type, and is not necessarily transferable to our particles. For instance Scherer, Robinson, and Kreuter [54] found that PBCA nanoparticles were almost unaffected by a high concentration of esterase the first two hours and Sommerfeld, Schroder, and Sabel [57] found that PBCA nanoparticles are unchanged the first 6 days in blood serum in vitro.

Student Vaksdal [66] did also an experiment with esterase. The result pointed in the direction that particles after 4 hours were more degraded in esterase than in just a buffer. However, after 24 hours a conflicting result was achieved, in which the remaining concentration had increased. It was suggested that the esterase somehow had aggregated and was counted as a nanoparticle mass.

2.4.6 Drug release

The drug is encapsulated to protect it from the surroundings, but also the other way around, to protect the surroundings from the drug. Nevertheless, the drug needs somehow to be released, and the main release of drugs happen either generally through diffusion or due to the surface degradation of the particles.[22, 47] Ideally the drug should be released in direct vicinity to the cancer cells or at best inside the cancer cells. If the drug is released by diffusion there is a great chance that some of the drug is released too early and at the wrong place. Hence, degradation that occurs in parallel with particle degradation is more predictable with respect to release rate and release destination.

The release profile for the drug is affected by several parameters, including the size of the drug and size of the nanoparticles, chemical structure and the nature of the delivery system. In the case of a polymeric matrix nanoparticle, the drug is uniformly distributed in the matrix and the release occurs by diffusion and/or erosion. The diffusion is affected by the size of the drug and the size of the polymer network. When higher molecular weight drugs are involved, diffusion through the polymer will be limited even though the drug is soluble in the external medium. If the diffusion is faster than the polymer degradation, the mechanism will then be diffusion dependent. Otherwise, it depends upon degradation.[47]

The main advantage of surface eroding polymers is the predictability of the erosion process. This is desireable when using polymers, where the release of drugs can be related directly to the rate of polymer erosion.

Due to the complexity of the particle degradation, a combination of diffusion- and degradation release is also possible, as reported for PLGA particles [30], in which an initial burst release based on diffusion is followed by a slower release upon degradation. Swelling is also used as a trigger for drug delivery [60], in which the space between polymers get bigger and the drug can easier diffuse through the polymer network. This is a field that need more research in order to understand the full mechanisms.

Since the PACA nanoparticle itself is hydrophobic, its purpose will mainly be as a carrier for hydrophobic drugs. A system will always try to minimize the energy of the system. A 'thermodynamic battle' will therefore decide how fast the drug is released from the particle. On one hand the drugs want to diffuse out of the particle in order to increase its entropy, and thereby reducing the total energy of the system. On the other hand a hydrophobic drug mixed with a hydrophilic solvent (here water) will be a very unfavourable energetic system. Hence, the strongest of these two contributions will decide whether or not the drug will diffuse out.[23, 45]

Westrøm [75] found in-vitro that NileRed, a hydrophobic fluorescent marker of the particles, is directly transferred to the hydrophobic cell membrane without first beeing dissolved in the hydrophilic cell medium. NileRed can be regarded as a model-drug, and its behaviour is in compliance with the theory above.

2.5 Particle stability

Stability is one of the most critical aspects of ensuring safety and efficacy of drug carrier products.

2.5.1 Sedimentation of particles

Sedimentation (particles sink) or creaming (particles float) is a problem that may arise in a nano-suspension, especially during storage. The rate of sedimentation and creaming for nanoparticles is given by Stokes' law (Equation (2.1)). It shows clearly the important role of the particle radius (R), the viscosity of the medium (η), the density of the particles (ρ_p)

relative to the density of the surrounding medium (ρ_f). v_s is the particle sedimentation velocity and g is the gravity constant. From the equation it is obvious that the easiest strategy to decrease sedimentation of particles is to reduce the size.

$$v_s = \frac{2}{9}\frac{(\rho_p - \rho_f)}{\eta} g R^2 \tag{2.1}$$

Equation (2.1) is the basis for Figure 2.10, in which the sedimentation velocity in cm per day is showed with respect to particle size and particle density.

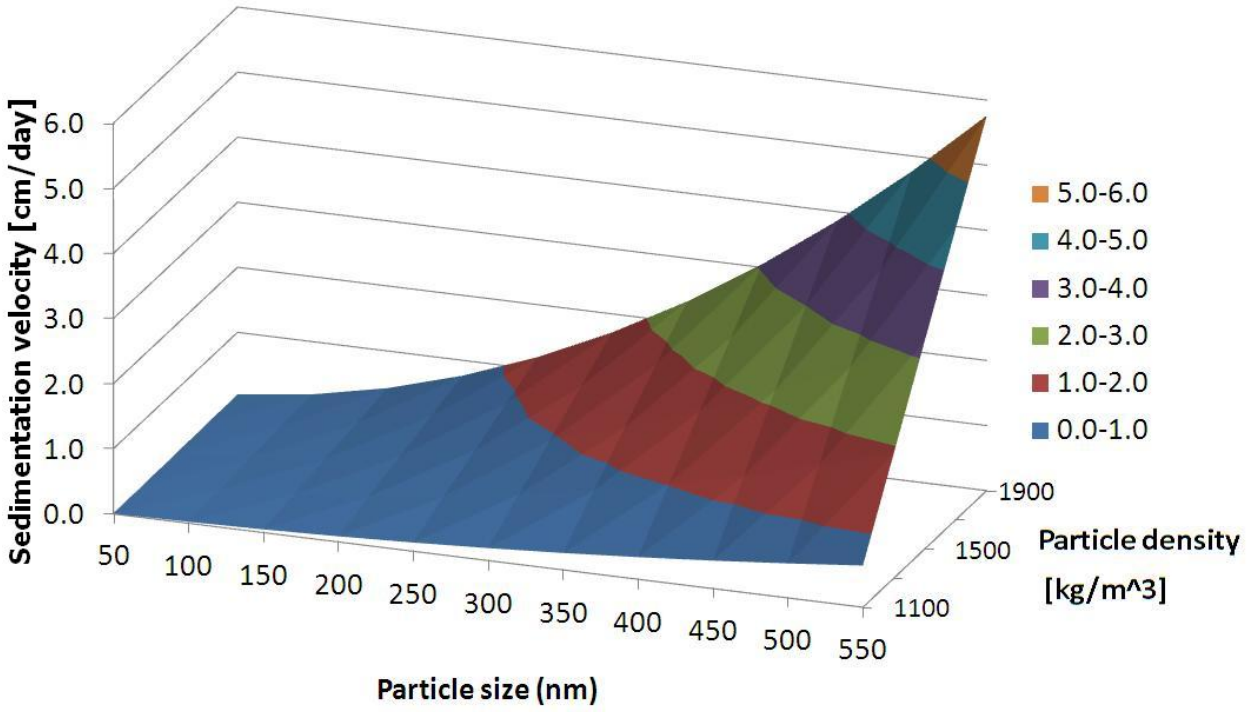


Figure 2.10: Sedimentation velocity [cm per day] for different particle sizes and densities. The density of other PBCA particles is found to be 1.148 g/ml (1148kg/m^3). [7] The density for our particle is likely approximately the same.

2.5.2 Minimizing the total energy

If the drug-carriers are not stable enough, there is a chance that the drug is released too early. Flocculated or aggregated particles[2] ($> 5\,\mu m$) can potentially lead to capillary blockades and embolism.[49] To be able to design nanoparticles that don't flocculate, one has to understand the mechanism influencing the stability.

[2]Flocculation and aggregation are two terms that are used around each other. Both are refering to the same system status, namely that particles are trapped in the secondary energy minimum (Figure 2.11), in which particles lay next to each other, but still have their own identity.

A system will always try to minimize its total energy ($\Delta G < 0$), and there are both stabilizing and destabilizing forces involved. As seen in Figure 2.11 there exist two energy minimima. The primary minimum corresponds to coagulation[3] of particles, in which they loose their identity completely. The secondary minimum which corresponds to flocculation is the situation where the particles lay directly next to each other, but without loosing their identity. A good example of the latter could be seen in Figure 4.15a. A system with nanoparticles is a very high energy system, because of the large surface area of the nanoparticles. This is thermodynamically unfavourable. Thus by reducing the total amount of surface, the total energy of the system will effectively be reduced. This is easily seen in equation (2.2), in which the total energy(G) of the system is given by the area(A) times the surface tension(γ). Hence, by reducing one of these two components the energy will be reduced. The system itself can usually not change the surface tension. Hence, the system will try to minimize the surface are and is doing this by organizing particles in aggreagates.

$$(dG = \gamma \cdot dA)_{T,P,n} \tag{2.2}$$

The main driving force for this to happen is the attractive Van der Waals-forces(VDW). They are acting between all particles and are very strong, but short-ranged. They are caused by electron fluctuations, which induce temporarily dipoles which can attract other induced dipoles. This force works as a constantly destabilizing contribution, and can lead to flocculation/coagulation.

However, there are also stabilizing effects which task is to prevent flocculation. The two main stabilizing effects are steric repulsion and electrostatic repulsion. The balance between the stabilizing and the destabilizing effects (Figure 2.11) determine whether or not the particles will flocculate, coagulate or stay stabile. [9, 23, 79]

Steric repulsion

Steric repulsion is a physical barrier between particles (Figure 2.12) which effectively hinder them to get close enough that the short-ranged VDW- forces can dominate. In addition to have a good solvation with the solvent, the steric layer has to be sufficiently long and dense to maintain a steric barrier that is able to screen the attractive VDW so much that they not any more are strong enough to cause flocculation. [79]

Electrostatic repulsion

A charged particle will in a solution be surrounded by ions and counter-ions in an electrical double layer(EDL). There are many models which describe this phenomenon, but the most commonly used is the combined Gouy-Chapman-Stern model.

It consist of two main layers, the Stern-layer which is a layer of immobilised counterions around the particle. The second layer is called the diffuse layer or Gouy-Chapman layer, where ions and counterions freely can move.

[3]Coagulation is the system status in the primary energy minimum in which particles melt together and loose their identity completely.

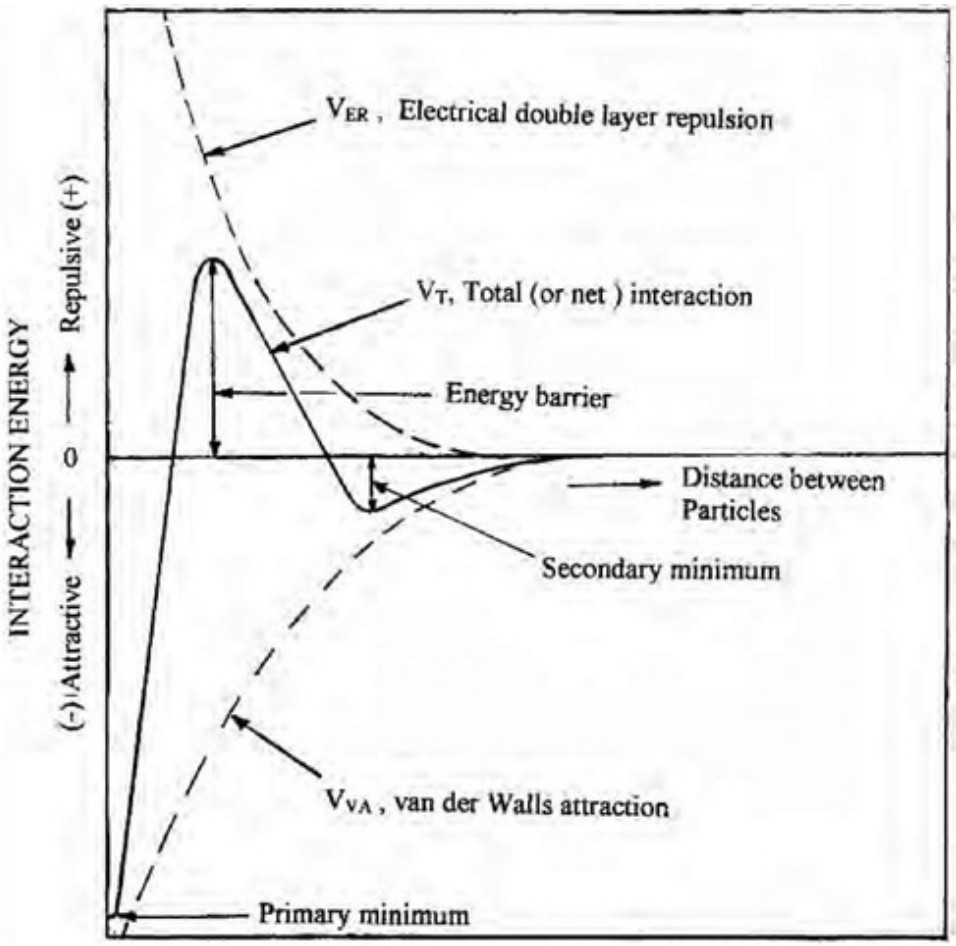


Figure 2.11: The stabilizing and destabilizing effects influence the stability of the system. The primary minimum reflects coagulation, whereas the secondary minimum reflects flocculation of particles. The steric contribution is not included in this figure

Since the particles have an immobilised layer of hydrated counter-ions around them, they behave as if they had a size with this hydration layer included. The Zeta-potential is defined as the electrical potential at the interface between immobile and mobile counter-ions (Figure 2.13). The zetapotential is an indicator of the stability of the particle, and a zetapotential of $|30mV|$ is generally believed to maintain stable particles. The Debye length(λ_D) is another important value, it is the length of the total EDL, and is given by equation (2.3), in which c is the ion concentration, Z is the valency of the ion k_B is the Boltzman' constant and T is temperature.

$$\lambda_D = \frac{1}{\kappa} \qquad \kappa = \sqrt{\frac{e^2}{\epsilon\epsilon_0 k_B T} \sum_i c_i^0 Z_i^2} \tag{2.3}$$

From this equation it is easy to see that the salt concentration has a great impact on the length of the EDL. The layer becomes screened and the electrical repulsion is very limited.

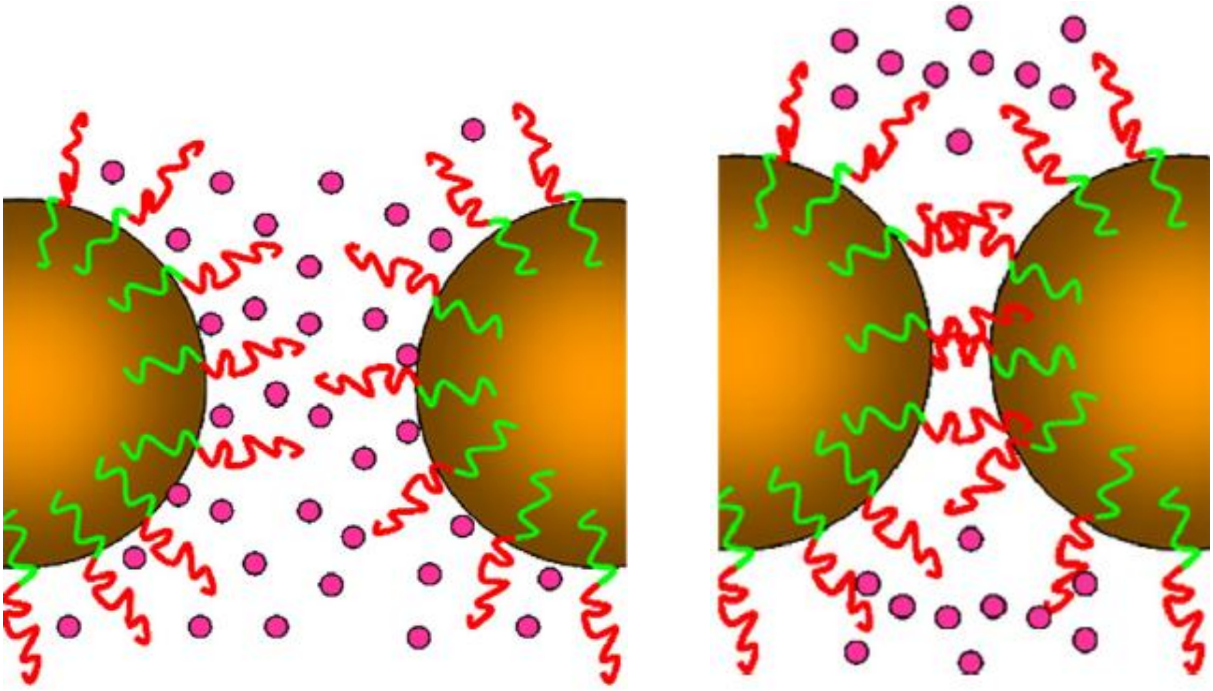


Figure 2.12: In the figure to left, the medium is a good solvent for the steric stabilizing molecules. Hence, the steric layers cannot interpenetrate to each other when the particles collide, which reduce the number of available configurations, which reduce the entropy. Hence, the particles will not flocculate. In the figure to the right, the dispersion medium is a poor solvent, the steric layer on the particles may then interpenetrate thermodynamically which induces particle flocculation. Image reproduced from Wu, Zhang, and Watanabe [79]

In blood there are so many ions that the Debye length is reduced to 0.78 nm (see Appendix 7.1 for calculations) [9, 13, 23, 79]

2.5.3 Proteins adsorption and PEGylation

Drugs and drug carriers needs a long circulation time, in order to stay in the vasculature system long enough to reach its desired target. When designing a drug carrier, it is not only the stability that needs to be in place. In the body the biological environment plays a significant role as well. It is therefore not enough to understand the chemical degradation of the particles, the biological removal of foreign objects is just as important.

Nanoparticles and foreign objects in general are rapidly removed by cells of the mononuclear phogocyte system(MPS). The MPS[4] [31] consists of dendritic cells, blood monocytes, macrophages in the liver, spleen and lymph nodes, in which all of them are responsible for clearing, processing and degrade foreign objects from the body.

Physiological environments like blood, cytoplasma and interstitial fluid contain hundreds of different proteins which contribute to the recognition of foreign materials. When a

[4]MPS was earlier called RES (Reticuloendothelial System), and this syntax is used in the reference.

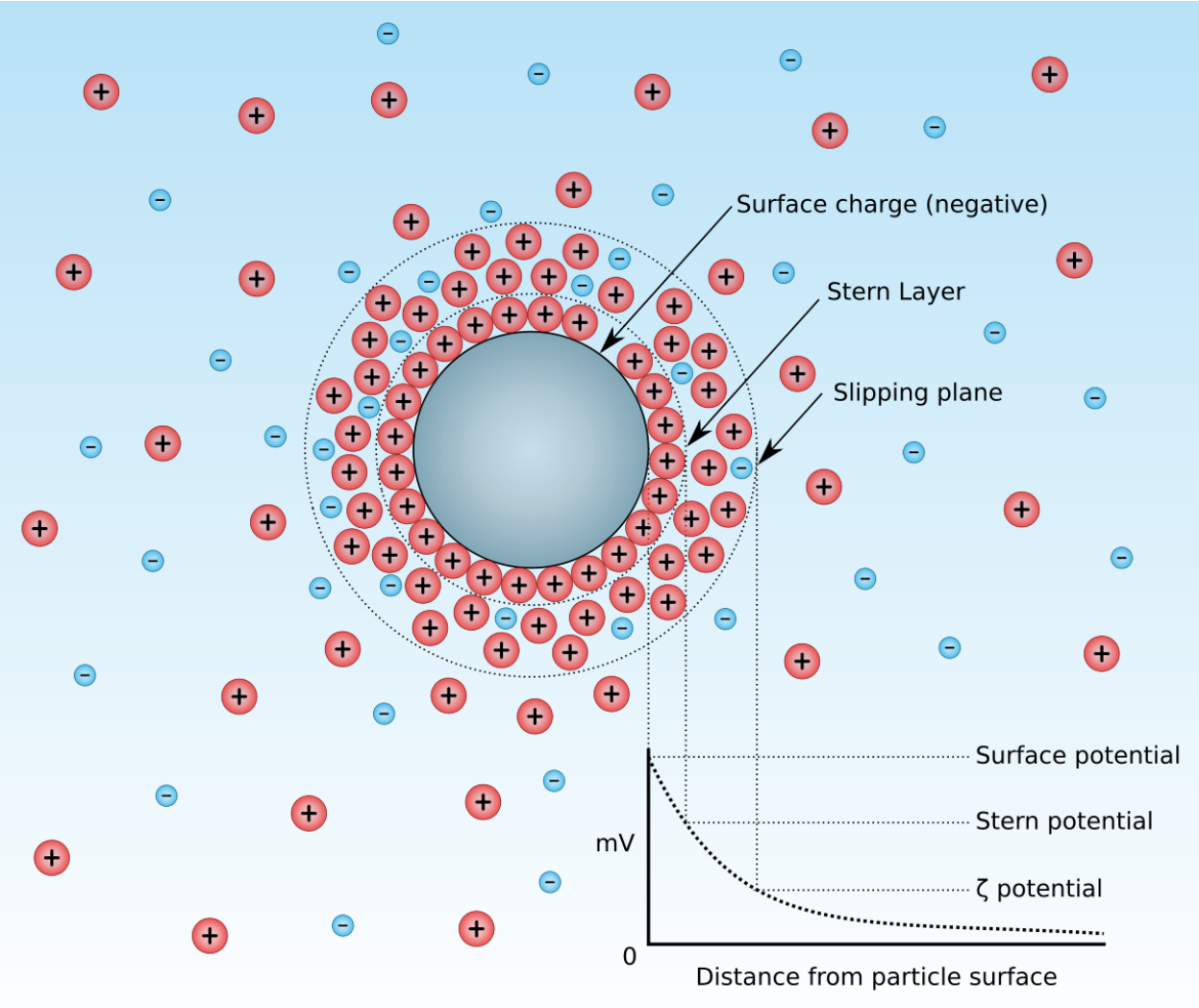


Figure 2.13: The electrical potential around a charged particle. Zetapotential is defined as the potential between the immobile ions and the free ions. Image reproduced from Wikipedia

nanoparticle enters these environments, proteins will immediately adsorb on the surface and form a so-called protein corona.[10] The protein corona changes the size, shape and surface chemisty, and the particle has now become its biological identity. With other words, the way biological components see it. The biological identity can be quite different from its synthetic identity (the way it is synthesized). Many of these adsorbed proteins will act as opsonins in the way that they tag particles which then can be recognized by the MPS system and finally be removed or destroyed.

In order to avoid this opsonisation process, the most prominent strategy today is to graft the particles with chains of polyethylene glycol(PEG) in a process known as PEGylation. PEG (Figure 2.14) is a nonionic, flexible and hydrophilic polymer. By attaching a PEG-layer on the nanoparticle surface the circulation time in body is signifi-

cantly increased.[31] It works by transforming a hydrophobic particle to a hydrophilic one. Additionally, it works as a steric hindrance for the proteins, which makes it harder for proteins to come in contact with the particle.

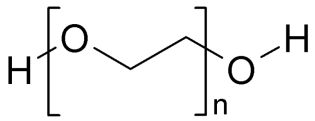


Figure 2.14: Polyethylene glycol

Recent studies with gold nanoparticles revealed that as much as 147 different proteins had adsorbed on the surface. It was additionally found that minimization of macrophage uptake does not require a complete elimination of serum protein adsorption. It was suggested that PEG minimized the macrophage uptake by selectively suppressing adsorption of specific serum proteins, and not by eliminating serum proteins completely.[72] With a high PEG density, the macrophage uptake is mainly driven by less efficient serum-independent mechansims (Figure 2.15).

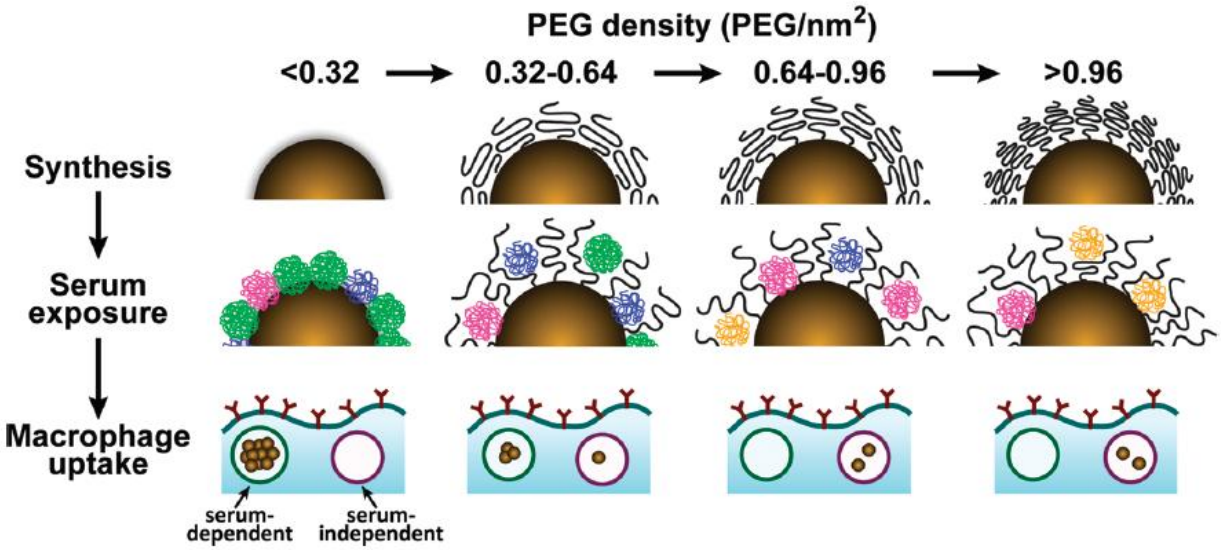


Figure 2.15: Schematic illustration of the influence of PEG density on serum protein adsorption and the macrophage uptake. At high PEG densities the macrophage uptake is shifted towards less effective serum independent mechanisms. Reproduced from Walkey et al. [72].

There are two main conformations of a PEG-layer. The *mushroom-* and the *brush-*layer (Figure 2.16). The PEG-density decides which conformations that is formed. If the density is high the brush-conformation forms and PEG-chains must stretch more in the vertical direction to fit, and a better steric hindrance is created. With a less dense layer the mushroom-conformation is formed, and each PEG-chain can stretch more freely in the horisontal way, which gives a less steric hindrance. To achieve the best shielding against immune cells and hydrolysis, the brush-layer is prefered.

All in all a long circulation time is essential in order to make an effective drug carrier. However, with a long circulation time there will also be a potential risk for damaging the healthy organs, because nanoparticles can end up in places where they not are supposed to,

and cause toxic reactions. This problem is the idea for introducing targeted nanoparticles. [2, 5, 31, 32]

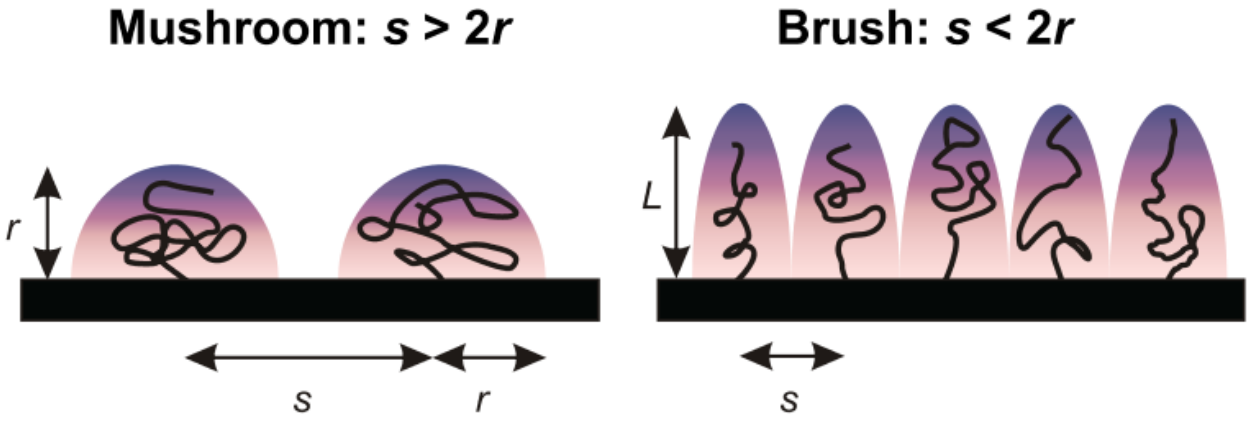


Figure 2.16: A description of the polymer conformation based on the grafting density. Reproduced from Backmann et al. [3]

2.6 The unique biology of a tumor and its advantageous for delivery of nanoparticles

The biology of a tumor is different from the biology in normal tissues. A tumor is associated with rapid growing cells, and thus the need for large amount of oxygen and glucose, which is transported by the blood vessels and diffuse to the cells. Since the diffusion length of oxygen is roughly 200 µm [21], growing cells will quickly come out of this diffusion range, and become *hypoxic*. Cells undergoing hypoxia releases survival- and angiogenic factors which trigger angiogenesis, the formation of new blood vessels. The production of new blood vessels happens very fast and in a uncotrolled manner, which results in a chaotic network of poor quality blood vessels. The gap between the endothelial cells of these blood vessels are larger than usual, which allow nanoparticles and other molecules in the same size range to leak through. This phenomenon, known as the EPR (enhanched permeability and retention)-effect, is utilized as a passive targeting of drug carrriers.

In addition to leaky blood vessels, hypoxia may lead to the *angiogenic switch*, which is a reorganization from an oxygen dependent metabolism, *glycolysis*, to a non-oxygen dependent metabolism, *anaerob glycolysis* (see Figure 2.17). In the anaerob glycolysis the glucose is converted first to pyruvate and then to lactate. This process is not very energy effective, producing only 2 ATPs in comparison to 38 ATPs for the normal glycolysis. To maintain the energy production the glucose delivery and the number of glucose transporters are increased, known as the *Warburg effect*. Another side effect of this system is increased production of H^+, and together with increased lactate production, this reduces the pH. This changed architecture and microenvironment upon hypoxia is the main contributions to the growth-

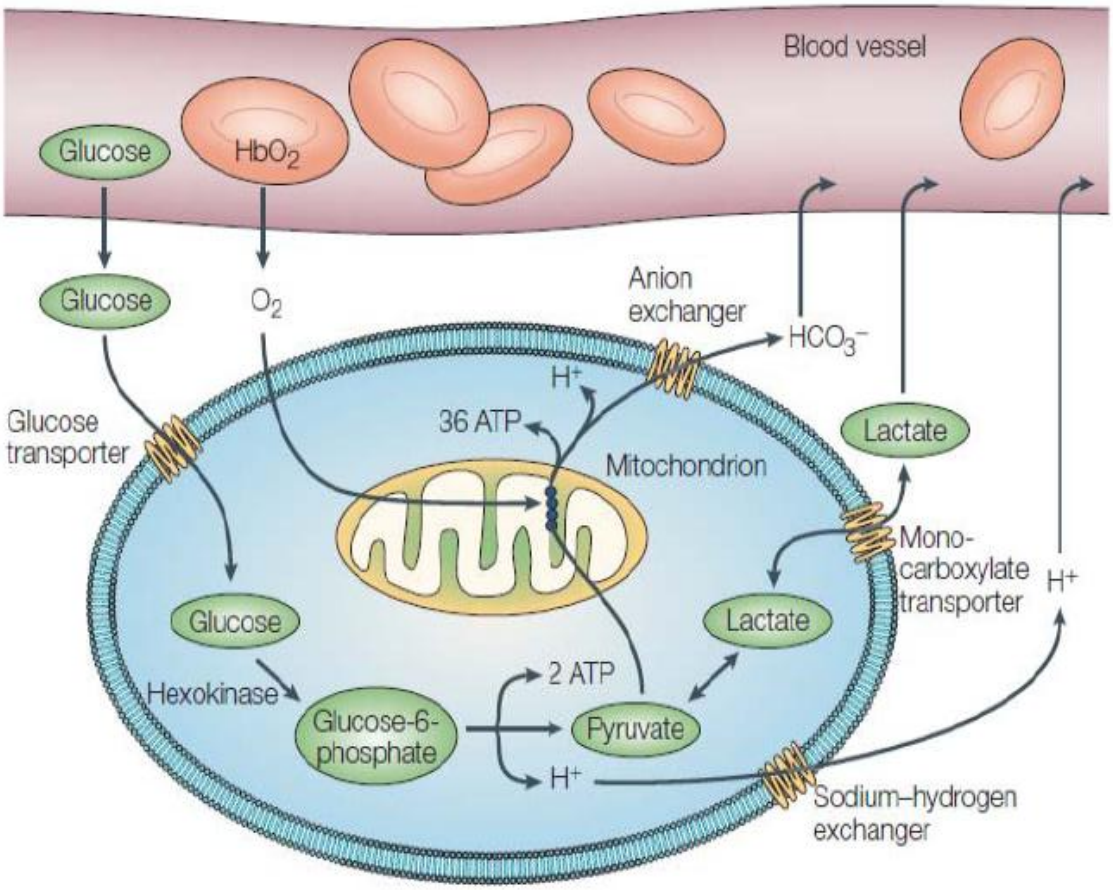


Figure 2.17: A schematic view of the anaerob glycolysis. Reproduced from Gatenby and Gillies [21]

induced stress, the abnormal vascular network, the elevated interstitial fluid pressure and the dense interstitial structure associated with tumor biology.

These biological differences can be utilized by the drug-carrier industry, for example by introducing pH-sensitive drug-carriers, or make particles that bind to receptors that are overrepresented in tumor environment, like the RGD-marked particle (see section 2.3.2)

[21, 73]

Physiological pH

The physiological pH is highly regulated and only a small deviation can be crucial for cells, proteins and enzymes. In arterial blood the pH is 7.38-7.42 and is regulated by a buffer-system including bicarbonate, ammonia and phosphate.[51]

As explained above, the microenvironment in tumors is generally more acidic than in normal tissue. How much the pH is reduced depends on tumor type, size and development-level.[62] Nevertheless, analysis have revealed that the pH can go as low as 5.8 for some

tumors.[76] This decreased pH will presumably have an effect on the degradation of the PBCA nanoparticles, since the degradation happens very slow in acidic environment.[57] This low pH may have a stabilising effect on the nanoparticle degradation, and the chance that nanoparticles remain intact until they have entered the cells increases. In the cells the pH is also close to 7.4, but in the lysosomes and endosomes, which is the place nanoparticles are believed to end up, the pH can be around 5.0 [5]

2.7 Instruments

2.7.1 Nanosight

Several techniques used to measure the size of particles utilize the light that particles scatter. This technique, however, require a priori knonowledge of the optical properties of the particles and the solvent in addition to camera sensitivity and performance. The Nanosight utilizes instead the tracking of Browninan motion of the particles in a liquid suspensions in the 10-1000 nm range (Figure 2.18)

A Nanosight-instrument contains a chamber where the particle solution is injected. The solution must be diluted to a particle number between 10^6 and 10^9 particles per ml. A laser illuminate the particles from aside, and the particles act as point scatters, whose dimensions are below the Rayleigh or Abbé limit. (The smallest resolution of an optical microscope) Despite the rapid movements of these particles, they can with help from the laser be tracked by a conventional CCD camera. A video is recorded and can be analysed analytically by a software program. The size of each particle can be separately determined and a quite accurate particle size distribution can be made. The movement of each single particle is followed and the mean square displacement is determined for each and one of them. The mean square displacement ($\overline{x^2}$) is related to diffusion (D) and time (t) through equation (2.4)

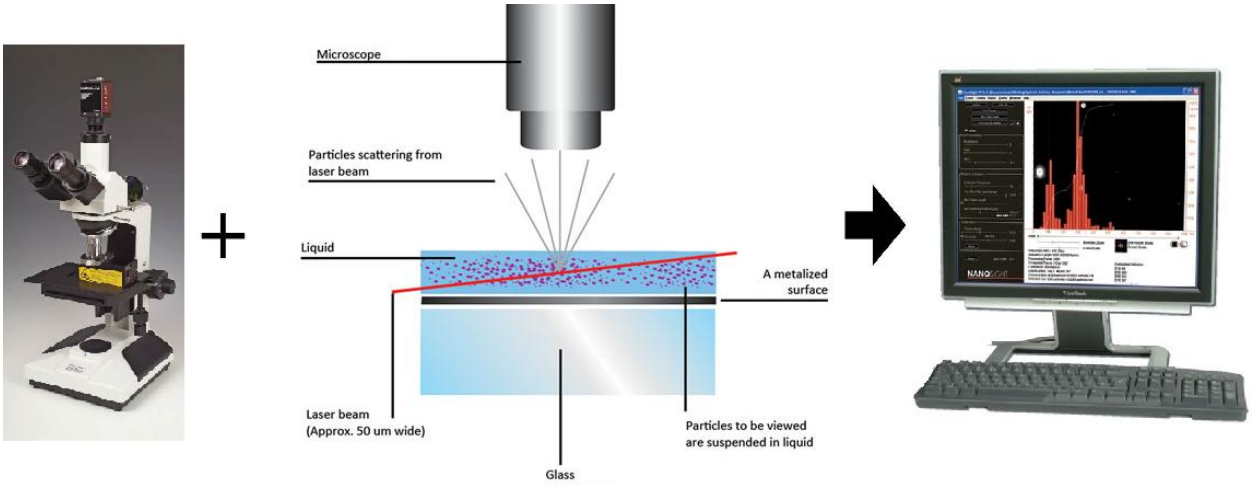


Figure 2.18: A graphical representation of the Nanosight principles. Images reproduced from `nanosight.com`

$$\overline{x^2} = 2Dt \tag{2.4}$$

From the Stokes-Einstein equation (equation (2.5)), the mean square displacement, the particle diffusion coefficient(D) and the hydrodynamic radius (r_h) are combined:

$$D = \frac{k_B T}{6\pi\eta r_h} \tag{2.5}$$

k_B is Boltzmann's constant, T is temperature and η is solvent viscosity. In addition to a very accurate size distribution, the Nanosight is able to calculate the concentration of the sample. In order to get an accurate number, it is important that the operator prepares the sample carefully and dilutes the sample to the right concentration, which is between 10^6 and 10^9 particles per ml. Ideally there should be approximately 100 visible particles in the visible window. A too high concentration will give poor result quality, because the software is programmed in the way that if two particles come too close to each other, the software cannnot distinguish them. Hence, the tracked path of both particles are deleted in order to avoid false tracking. This in turn leads to a lower concentration than what it actually is. On the other hand, if the number of particles is too low, the statistical data will be to small to get a good analysis.

The Nanosight software generate a particle distribution. The width of this distribution is given in STD or standard deviation (Figure 2.19). Within one standard deviation (represented by σ) of a particle distribution, 68.2% of the datapoints are collected. The standard deviation is defined as the square root of the variance of a distribution.[58]

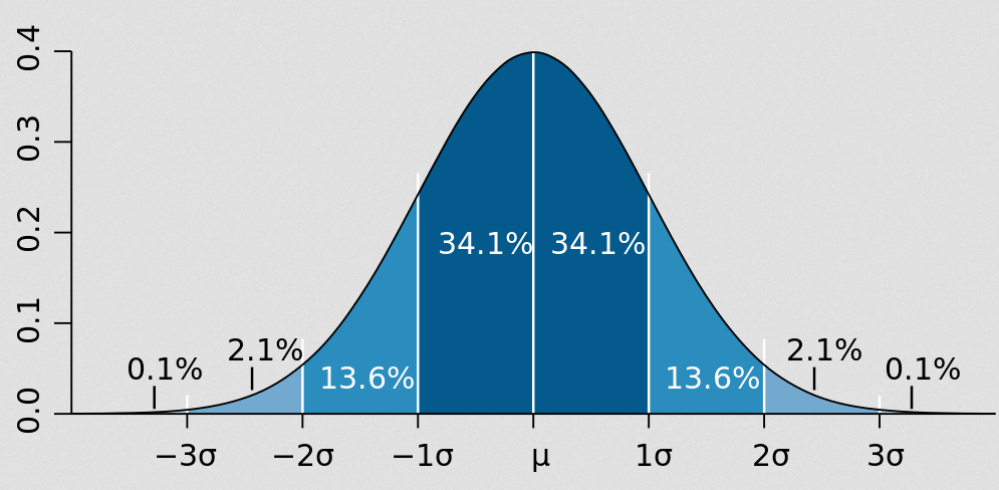


Figure 2.19: The standard deviation gives the width of a particle distribution. Image reproduced from Wikipedia.

A Nanosight instrument has several parameters that must be manually chosen by the operator, see Table 2.1.

Table 2.1: Parameters of the Nanosight

Parameter	**Function**
Shutter	determines the time the camera shutter is open.
Camera Gain	regulates the sensitivity of the camera.
Capture Duration	determines the length of the captured video. A too short video may result in inaccurate and statistically poor size distributions
Gain	adjust the intensity of the scattered light from the particles, and thereby affect how many particles that are able to track. With a high gain many more particles become visible for the detector.
Blur	eliminates noise and smoothen the shape of the particles.
Detection Threshold	determines the minimum grey scale value of a dot necessary to be tracked.
Minimum Expected Particle Size	determines the area around the particle in which the software searches for it in the following frame. Large particles have slow movements, whereas small particles have fast movements.

[39]

The Nanosight instrument generate a pdf with the main results. A few of these are printed in the Appendix as an example.

2.7.2 Zetasizer

A Zetasizer is the most user-friendly and most used instrument for analysis of nanoparticles and protein aggregates.[18] It has the ability to measure three fundamental parameters, namely particle size, zeta potential and molecular weight. The zetasizer calculates the size by first measuring the Brownian motion of the particles in a sample using Dynamic Light Scattering. This is done by illuminating the particles with a laser and analysing the intensity fluctuations in the scattered light (Figure 2.20). Equally sized particles will contribute to a constructive interference and the intensity measured will be stronger. As the particles move the intensity signal will fluctuate. The signal wil fluctuate faster for small particles since they are moving faster. The calculated Brownian motion is then combined with the Stokes Einstein equation (equation (2.5)) and the correlation function is then utilized to find the size distribution.

One should be aware that the the mean size is an intensity mean. It is not a mass or a number mean because it is calculated from the signal intentsity. The intensity of the scattered light is proportional to the sixth power of the particle diameter, which makes this technique very sensitive to the presence of large particles. If the purpose is to detect small quantities of large particles this is a very valuable technique. On the other hand, it makes it very hard to detect an accurate size in a polydispersive sample. Therefore if the polydispersity index is high, one cannot trust the results.

The polydispersity index (PDI) is defined as the square of the standard deviation/mean particle diameter (Equation (2.6)). For example, a particle with diameter 100 nm and a PDI of 0.1 would have a standard deviation of 31.6nm. Particles with a PDI less than 0.1 are typically referred to as monodisperse.

$$PDI = \left(\frac{\sigma}{d}\right)^2 \tag{2.6}$$

[18, 29, 44]

2.7.3 Gas Chromatography

Gas Chromathography (GC) is an instrument (Figure 2.21) used a lot in analytical chemistry for quantitative analyses of compounds that can be vaporized without decomposing. The sample to be analysed must be mixed with a highly volatile solvent, for example diethylether. If the sample originally is a water-based systems, the actual substances must first be extracted out. An internal standard is added to the sample in order to better control the outcome. The liquid sample is then heated to a vapor and is pushed through a column by a carrier gas. The motion is inhibited by the adsorption of the analyte molecules onto the column walls. Different molecules will interact differently with the wall of the column based on their molecular composition. Since each type of molecule has its own unique progression time, they will enter the end of the column at different time intervals, called the retention time. When passed the coloumn, each molecule is registered and quantified by a sensor and this makes it possible to differentiate between many different substances in one mixture. [63]

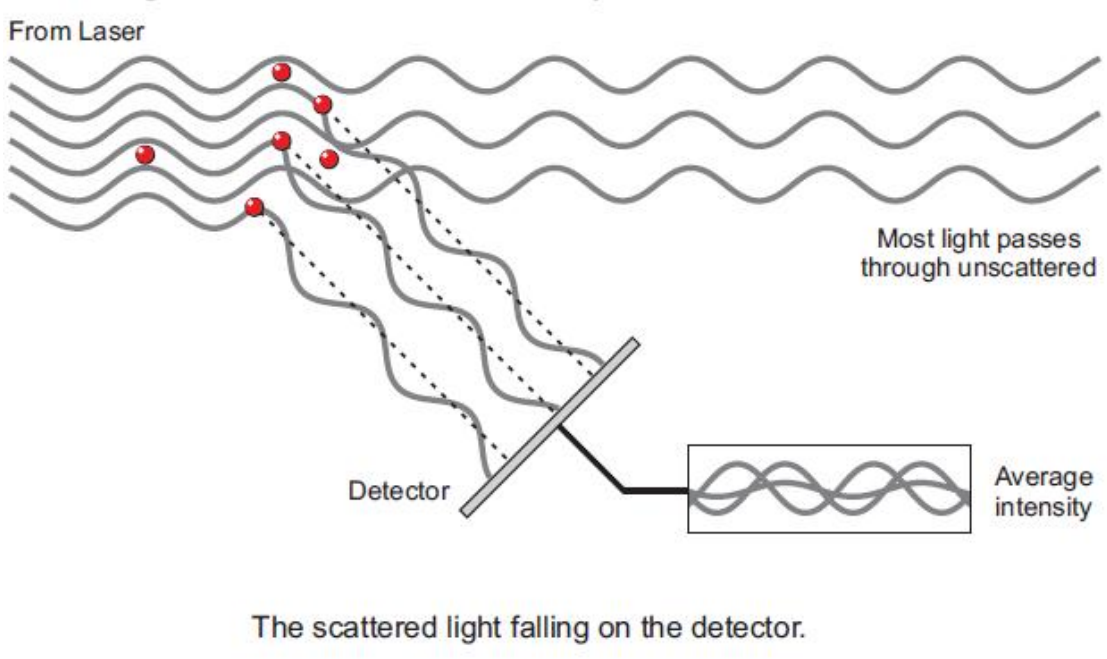


Figure 2.20: Light scattering of particles is the principle of the Zetasizer

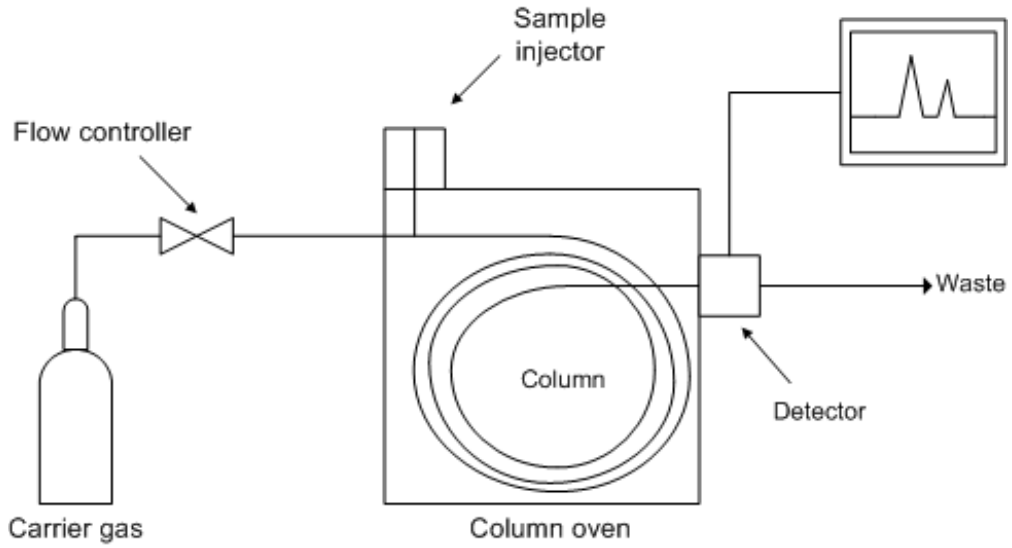


Figure 2.21: A schematic illustration of a Gas Chromatography-instrument. Image reproduced from Wikipedia

Theoretical maximum of butanol and octanol

Since the concentration of nanoparticles in the stock solution is known, it is possible to calculate the theoretical maximum amount of degradation products. The stock solutions are for PBCA 56mg/ml and for POCA it is 42mg/ml. The molar masses of the components are:

$$M_W(BCA) = 153.12\frac{g}{mole} \qquad M_W(butanol) = 74.12\frac{g}{mole},$$

$$M_W(OCA) = 209.28\frac{g}{mole} \qquad M_W(octanol) = 130.23\frac{g}{mole}.$$

During the degradation, each monomer-molecule can generate one molecule of an alcohol (butanol or octanol).

That means that the theoretical butanol-outcome for PBCA is $\frac{74.12g/mole}{153.18g/mole} = 48.4\%$

and for octanol it is $\frac{130.23g/mole}{209.28g/mole} = 62.2\%$

If 5ml of a 1:250 dilution is used, it means that the nanoparticle concentration becomes $\frac{56mg/ml}{250} = 0.224mg/ml \rightarrow 0.224mg/ml \cdot 5ml = 1.12mg \rightarrow 1.12mg \cdot 48.4\% = 0.54mg$

$\rightarrow \frac{0.54mg}{74.12g/mole} = 7.3 \cdot 10^{-6}\, mole$

which is the maximum theoretical amount of butanol from 5 ml of 1:250 PBCA solution.

$\frac{42mg/ml}{250} = 0.168mg/ml \rightarrow 0.168mg/ml \cdot 5ml = 0.84mg \rightarrow 0.84mg \cdot 62.2\% = 0.52mg$

$\rightarrow \frac{0.52mg}{130.23g/mole} = 4.0 \cdot 10^{-6}\, mole$

which is the maximum theoretical amount of octanol from 5 ml of 1:250 POCA solution.

This calculation, however, assume that the polymer is 0% degraded when weighed, and that ester-hydrolysis is the only degradation pathway.

2.8 How to mimic a physiological environment and the choiche of degradation pHs

It is hard to make a system that mimic the body completely. The body is a highly complex dynamic system, in which fluid, salts and lots of other compartments are constantly exchanged. The degradation of particles will be affected by all these processes, and since an understanding of how the particles will degrade in a physiological environment is the main purpose of these testing, it is naturally that the testing-setup is somehow comparable to the conditions that is found in the body. To make an identical setup is impossible, so a few compromises must be made.

When developing a test-method it is important that it starts pretty simple, perhaps with only one changing parameter, in order to understand how it affects the process. Later testing can be made more complex, involving for instance animal testing. Finally testing in a human body can be made.

Since the degradation is affected by the pH. It is interesting to see how particles behave when exposed to different pHs. As mentioned the pH is quite constant in the body, but is still varying along the path of the nanoparticle. In the blood for instance the pH is close to 7,4.[51] The pH of the tumor interstitium can be slightly lower(5.8-7.4)[76], dependent on tumor type, degree of vascularisation and level of development.[67] In the cells the particles will probably end up in the lysosomes where the pH is approximately 5.[5] The setup for buffer testing is three different pHs: 7.4, 5.5 and 4.0. The latter is additionally added because the particles are stored at this pH.

Chapter 3

Materials & Methods

3.1 Development of a method for degradation

As mentioned in theory a part of the project was to develop a method for degradation. The original idea for this project was therefore to prepare diluted nanoparticle solution in different mediums and measure how the mean size, the size distribution and the zeta potential varied over time. The size is interesting to observe because surface erosion and particle size decrease is reported in the litterature. Polydisersity index (PDI)[1] is relevant to see if the particles stay monodisperse or not. The zetapotential is interesting because of stability issues and the degradation should in theory make negatively charged groups, which may change the zetapotential.

3.1.1 Particles used

The particles used in this project are poly butylcyanoacrylate, known as PBCA, and poly octylcyanoacrylate, known as POCA. They are produced by SINTEF Materials & Chemistry through a miniemulsion technique (see section 2.3.2). The density of particles in the stock solution is 56 mg/ml for PBCA and 42 mg/ml for POCA.

3.1.2 Mediums used

A water solution and three different buffers were prepared. A 0.01M pH 7.4 phosphate buffer was made my mixing 1.20g disodium phosphate and 0.312g monosodium phosphate in 1 litre of water. The 0.01M pH 5.5 was made by mixing 0.09g acetic acid and 1.16g sodium acetate in 1 litre of water. 0.01M pH 4.0 was obtained by adding 0.51g Acetic acid and 0.21 g sodium acetate in 1 litre of water. The buffers were measured by a pH-meter and adjusted to the right value by adding more of either acid- or base-component, if they differed from the right

[1]The Zetasizer gives the PDI, whereas the Nanosight gives standard deviation(STD). However, both are a measure of the width of the sample distribution, and they are combined through equation (2.6)

value. The different pHs were chosen to simulate various physiological environments (see section 2.8).

Additionally, the particles were tested in cell medium (see Appendix for specific information), in blood serum, brought from the Molecular medicine-department at St. Olavs hospital, and in esterase buffer solution. Esterase tests are earlier carried out with a concentration of 220-880 units/ml. [61] One unit will hydrolyze 1.0 μmole of ethyl butyrate to butyric acid and ethanol per min at pH 8.0 at 25°C. For our experiment porcine liver esterase was bought from Sigma Aldrich and had a sensitivity of 17 units/mg. 190mg for 5ml (646 unit/ml) was therefore chosen as an appropriate value. The esterase was added to a pH 7.4 phosphate buffer.

3.2 Setups

To different setups (Figure 3.1) are used for degradation.

3.2.1 Dialysis-cassette

The degradation setup consist of a nanoparticle-buffer solution placed in a dialysis cassette[2], which is placed in a buffer bath. This setup is supposed to be a simple model of the physiological environment. With dialysis the medium is constantly exchanged, the degradation products are removed, whereas nanoparticles stay in the solution. This system ensure that the degradation does not reach an equilibrium. For pH 7.4, pH 5.5 and pH 4.0 a 1:300 dilution was tested. For the pH 7.4 buffer dilution a 1:500 was additionally tested. This to see if a more diluted concentration had an effect. A 1:300 dilution corresponds to 0.187 mg/ml and 0.14 mg/ml for PBCA and POCA, respectively. And 1:500 to 0.112 mg/ml and 0.084 mg/ml.

3.2.2 Reagent bottles with and without rotation

Another setup consisted of nanoparticles in 15 ml large reagent bottles. The bottles were placed in an oven at 37°C without rotation. Particles were mixed in different mediums like buffers pH 7.4, cell medium, blood serum and esterase. This setup worked fine for all samples except for the esterase sample, which behaved quite differently. It sedimented within few hours. Hence, after approximately 24 hour, the esterase-bottles were installed in a rotating device, and no more sedimentation was seen. The concentration used here was 1:250. It should have been 1:300 as the one for the dialysis cassette, but due to a calculation mistake it became 1:250.

[2]The dialysis cassette is bought from Thermo Scientific and has a molecular weight cut off of 20.000.

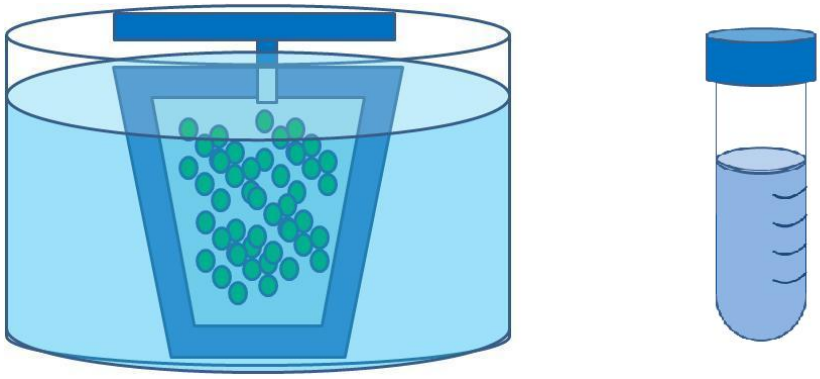


Figure 3.1: To the left the dialysis cassette can be seen. It contains 28 ml of 1:300 solution of nanoparticles. The cassette was placed in the same buffer, approximately 400 ml. The buffer was replaced every 3-4 days. To the right a 15 ml reagent bottle can be seen. This is a very simple test system, in which no exchange of fluid happens. On the other hand, this bottle can easy be placed in an oven and installed on a rotating device.

3.3 Zetasizer-measurements

The Zetasizer (from Malvern Instruments) was used to find the mean size, the polydispersity index and the zetapotential. The samples do not have to be diluted or prepared in another way before the measurements. Experimentes were ended after a week because results were not reproducable. No more test were carried out on the Zetasizer instrument. Instead the Nanosight was chosen.

3.4 Nanosight-measurements

PBCA and POCA nanoparticles in different mediums were measured by the Nanosight. Two different setups for degradation were chosen. The first setup was the dialysis cassette, it was carried out on the different buffer systems with dilution 1:300 and 1:500. Here 1 ml of the buffer solution was mixed with 33 or 40 ml of deionized water, with a final dilution of 1:10 000 (5.6 $\mu g/ml$ and 4.2 $\mu g/ml$ for PBCA and POCA, respectively).

The second setup was the reagent bottle-method, which were carried out for the different mediums. These were prepared with 20 µl nanoparticle solution (1:250) and diluted with 5 ml of deionized water, to a final dilution of 1:12500. (4.5 $\mu g/ml$ and 3.4 $\mu g/ml$ for PBCA and POCA, respectively) Ideally the samples should have been diluted with the same medium, but the amount of medium was too limited for this.

The first measurement was always carried out within 5 minutes after mixture to ensure that no degradation had taken place before this measurement. Directly after measurement the 1:250-sample was then placed back in an oven at 37°C. The next measurements were performed almost identically with the first one, except that a vertex rotator was used for some seconds to ensure a homogeneous concentration of particles before the measurement.

The Nanosight has many different parameters (see section 2.7.1). All these parameters

where carefully chosen to get the best results, and for each sample parallel these parameters where identically chosen to minimize measurement inaccuraties.

3.5 Gas Chromathography setup

Gas Chromatography (GC) was used to detect and quantify the degradation products (butanol and octanol) of PBCA and POCA nanoparticles in buffer pH 7.4. The samples must be centrifuged in order to remove the nanoparticles. This was carried out in accordance with earlier GC-tests. [61]. The samples were rotated in a super centrifuge for 20 000 RPM in 1 hour at roomtemperature. A volume of 30 ml per sample was chosen because the centrifuge rotor only could handle those amounts. Thereafter 5 ml of the supernatant was removed and. 3.0 µl of n-pentanol was then added to the supernatant, to get an internal standard for the measurements. 5 ml diethylether was added to the supernatant and the mixture was shaken thoroughly in order to extract the alcohols. GC-sample bottles were then filled completely to the top and analyzed. A RTX-1 capillary column was used with helium as the carrier gas. For measurement of the butanol the oven temperature was isothermal for 3 minutes at 45°C and then ramped to 130°C at rate 10 degrees/min and held at this temperature for 3 minutes. For octanol the oven temperature was isothermal for 3 minutes at 45°C and then ramped to 200°C at rate 10 degrees/min and held at this temperature for 3 minutes.

A test with butanol, pentanol and octanol was carried out to find the exact retention times. A complete degradation test of butanol and octanol was carried out, in which nanoparticles were mixed with a 0.05M NaOH solution (1:250) and stored in an oven at 37°C with 5 rpm rotation for 10 days. This was carried out to get a complete hydrolysis of the particles and thereby get a sample with maximum amount of butanol and octanol respectively.

The butanol/octanol yield is then calculated by comparing the amount of butanol/octanol to the maximum amount of butanol/octanol from the fully degraded sample. The mass of the butanol and octanol can be calculated by comparing it with the known amount of an internal standard, which here is pentanol.

Finally, three test series were prepared. PBCA 1:25(2.15mg/ml), PBCA 1:250(0.22mg/ml) and POCA 1:250 (0.17 mg/ml) in a pH 7.4 phosphate buffer solution. They were prepared at different time intervals and stored in an oven 37°C. The reason why they were prepared with different time intervals was simply to get different degradation lengths. After a given time they were prepared and measured as described above.

3.6 Generation of Nanosight-plots

In the Results-chapter, several curves from the Nanosight measurements are shown. These curves are made from the pdf-file which is generated after a Nanosight-measurement. In the Appendix, examples of these pdfs can be seen. In some curves there are error bars, these curves are made on basis of three paralell samples. The plotted point is the average from

these 3 paralells and the error bar show the highest and lowest value within these paralells.

3.7 Instrument details

Zetasizer	
Type:	Nano ZS90
Manfacturer:	Malvern Instruments
Laser:	633 nm

NanoSight - Nanoparticle Analysis System	
Type:	LM10HS
Laser:	635 nm
Objective:	Plan-Apochromat 63x/1.4 oil DIC M27
Camera:	EMCCD (Electron Multiplication Charge Coupled Device)
Resolution:	30nm-1000 nm
Concentration:	$10^6 - 10^9 \, per \, ml.$
Capture Time:	60 seconds
Completed tracks:	1300-13 000
Frames per second:	30

Gas Chromatography	
Type:	Agilent 7890A Gas Chromtograph
Column:	RTX-1
Solvent:	Diethylether
Start temperature:	45°C
Slope:	10°C/ min
End temperature:	240°C
Split:	1:5
Injection volume:	1µl

Scanning Electron Microscopy	
Type:	Hitachi S-5500
Acceleration voltage:	15kV
Current:	10 µA
Sensor:	Secondary electrons gave the best results.
Coating:	10 nm gold

Chapter 4

Results

4.1 Nanosight - Degradation of PBCA and POCA in buffers with different pHs

As mentioned in the theory (section 2.4.3) the pH is believed to affect the degradation process. In order to find this relation, experiments were carried out on three buffer systems with different pHs, namely pH 4.0, pH 5.5 and pH 7.4. Two nanoparticle types with different alkyl chains are tested, PBCA and POCA.

4.1.1 Behaviour of PBCA particles in different pHs

A clear trend was seen for the degradation in the three different buffers. The degradation of nanoparticles was more pronounced at a higher pH, as shown in Figure 4.1 and Figure 4.2. The concentration of particles in buffer pH 4.0 decreased slightly the first 20 hours, then it was stable. After 13 days (300 hours) the particle concentration in the pH 4.0 buffer was still 90%, whereas it was approximately 35% for pH 5.5.

For PBCA pH 7.4 several measurements were carried out: (1:250, 1:300 and 1:500) The concentration decreased fast for all of them and the mean size incresed. These results can be seen in Figure 4.3 and Figure 4.4. For the 1:250 sample the concentration has decreased to 63% after 3.5 hours (average of three samples), whereas 47% is reached after 8.5 hours, and 30% after 24 hours. The initial size is 143 nm, after 3.5 hours 206 nm, 234 nm after 8.5 hours and 256 nm after 24 hours. This concentration decay was the fastest for all mediums, but after 8 days the concentration was the same as for 1:300 and 1:500.

A curve fitting in Matlab was carried out for PBCA in order to see if the degradation could be related to some kind of mathematical relation (Figure 4.5). For pH 4.0 the concentration decrease was so little, and this could be described relatively good by a linear function. pH 5.5 and 7.4 seemed to be proportional to a $\frac{1}{x}$ decay, in which the decay was very fast the first hours, and then a more gradual decrease was seen.

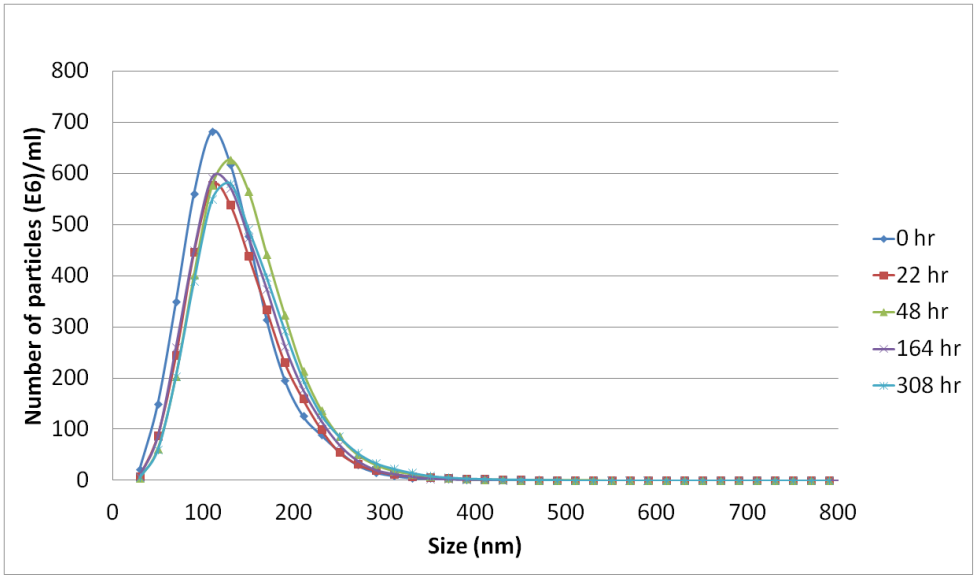
From the particle size distributions of 1:500 and 1:250, one can see that the number of small particles (<180 nm) was reduced over time, whereas the number of large particles (>180 nm) increased. This made the STD (standard deviation) of the particle size distribution to increase.

After 8 and 13 days, something interesting happened for PBCA pH 7.4 1:300 and 1:500. A fast reduction in size was observed. Especially for 1:500, this change was unambiguously. From day 8 to day 10 the mean size dropped from 286 nm to 153 nm, and between day 10 and day 14 it was reduced even further to 115 nm. For the 1:300 sample the drop was not that big, but it was still clearly visible. From day 13 to day 17 the size has fallen from 216 nm to 136 nm. This sudden drop was not observed for other samples, but none of the other samples were measured for that many days.

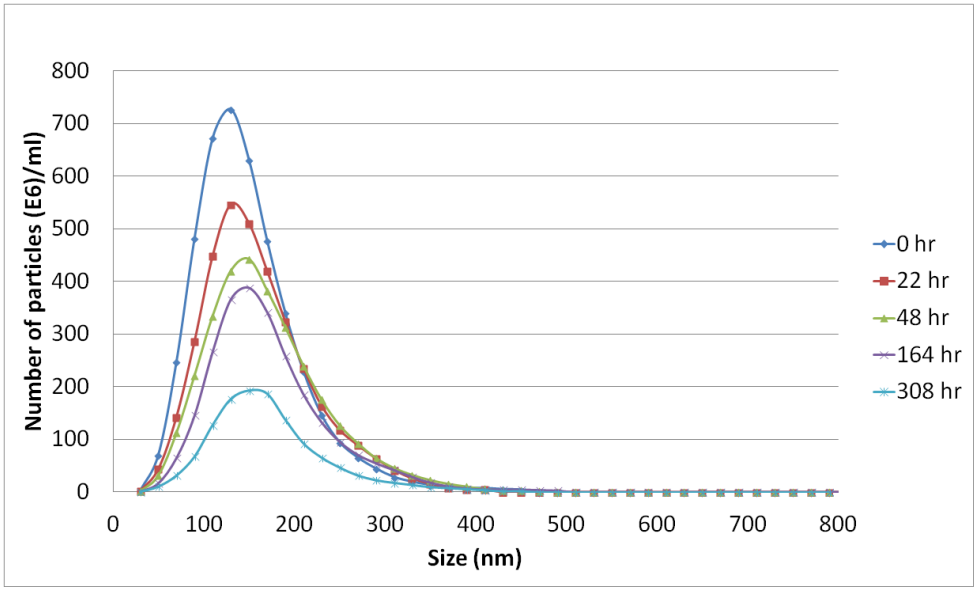
4.1.2 Behaviour of POCA particles in different pHs

In contrast to the PBCA particles, the degradation of POCA particles seemed to be unaffected by pH differences, as seen in Figure 4.6 and Figure 4.7. As a matter of fact, they behaved almost identical for all the three different pHs. Some small differences were there, but not more than what one could expect as instrument-inaccuracies. The concentration has been reduced to about 75% within the first 20 hours, then the concentration fluctuated a bit before it increased again. After 13 days the size has increased from around 160 to 170 nm, whereas the STD of the samples was almost unchanged

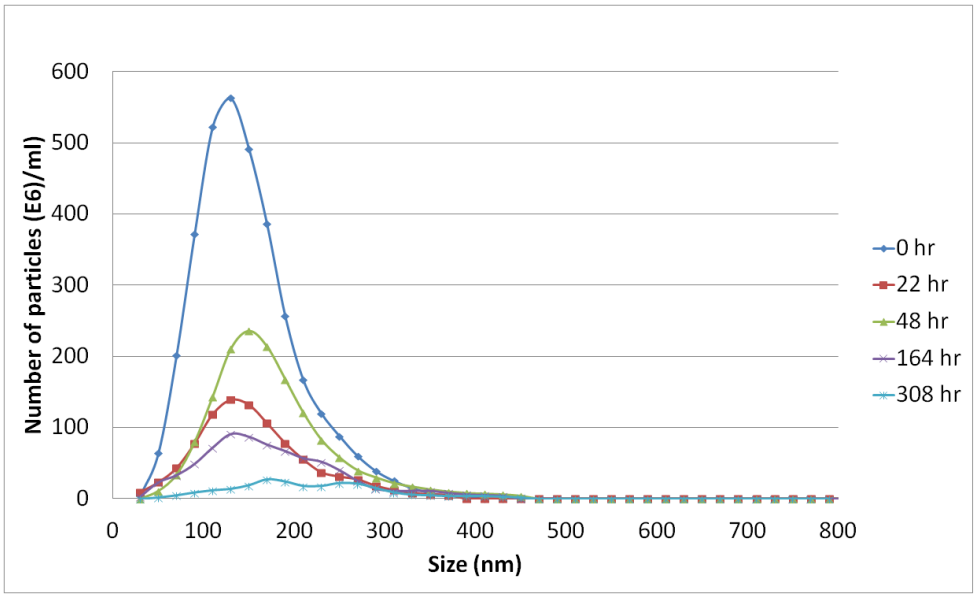
Based on these experiment, there was a clear difference between PBCA and POCA particles. POCA particles were much less degraded and turned out to be little affected by the pH. Degradation of PBCA particles, on the other hand, were highly influenced by the pH, in which a higer pH gave a faster degradation.



(a) Particle distribution of PBCA buffer pH 4.0.

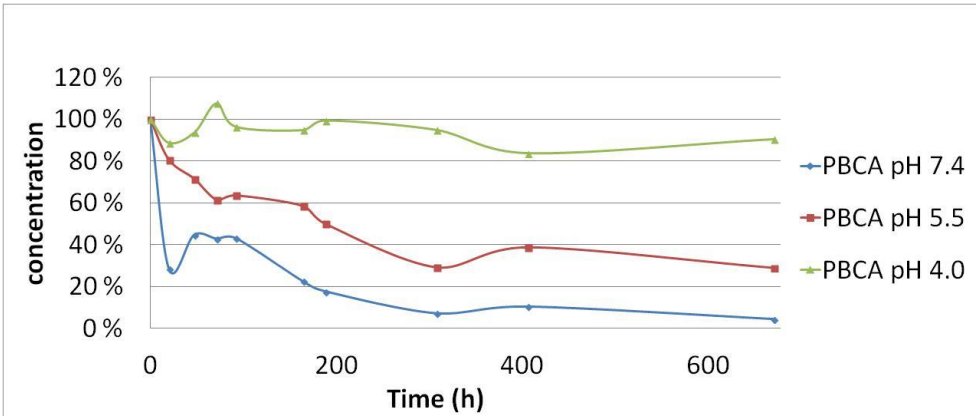


(b) Particle distribution of PBCA buffer pH 5.5

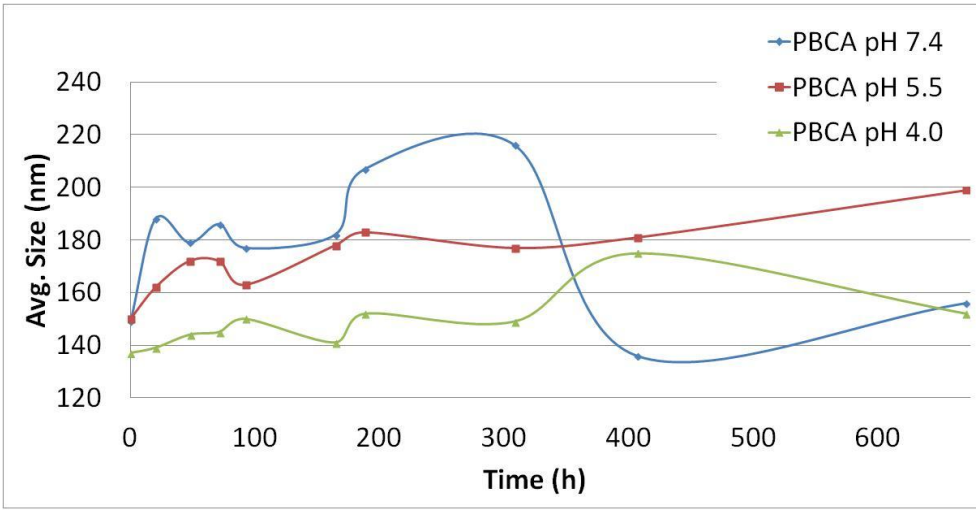


(c) Particle distribution of PBCA buffer pH 7.4

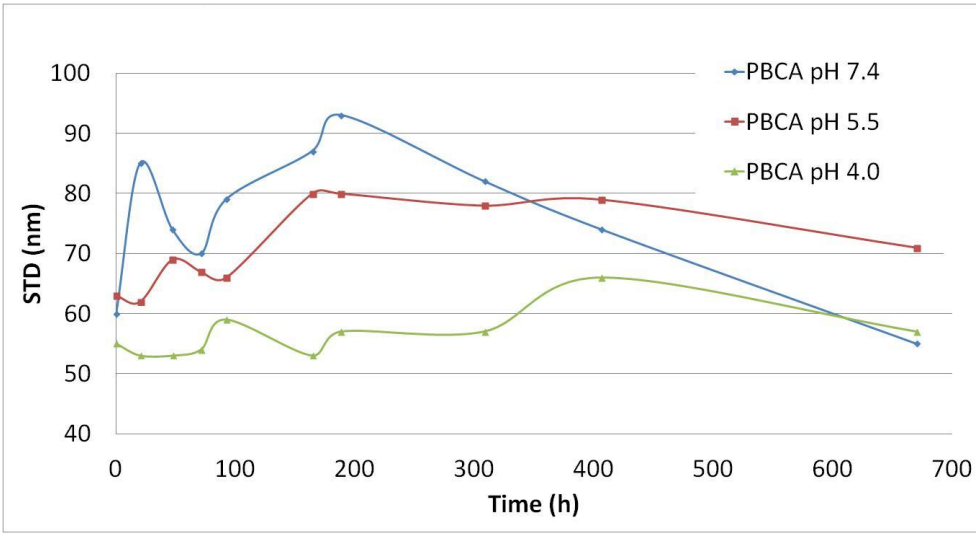
Figure 4.1: Particle distribution of PBCA in different buffers. This is the the same particles as in Figure 4.2. All samples are diluted to a 1:300 concentration, and the dialysis-cassette setup is used.



(a) Concentration decay for PBCA nanoparticles 1:300 in different pHs .

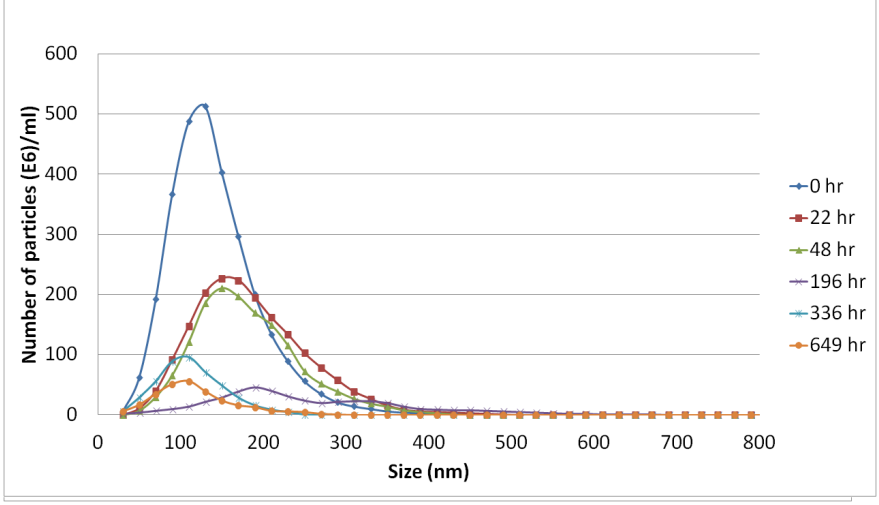


(b) Size change for PBCA nanoparticles 1:300 in different pHs

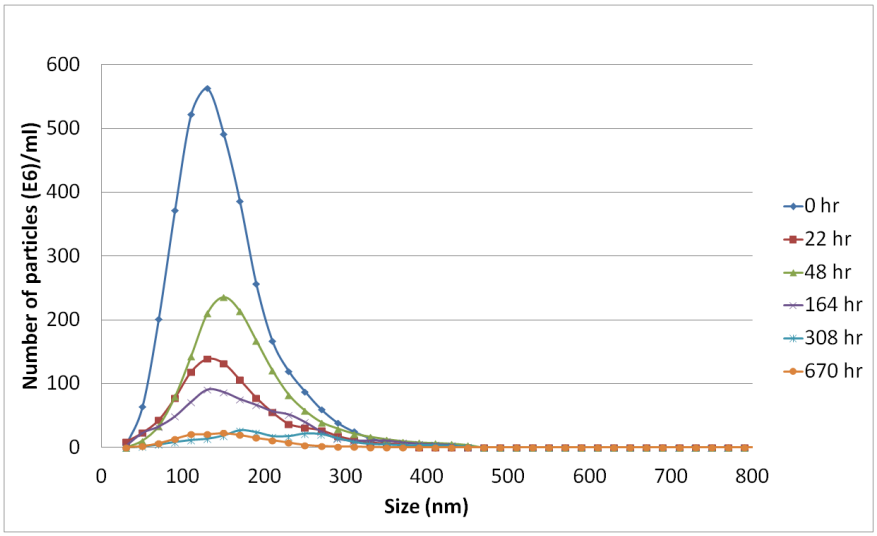


(c) Width of particle distribution of PBCA buffer pH 7.4

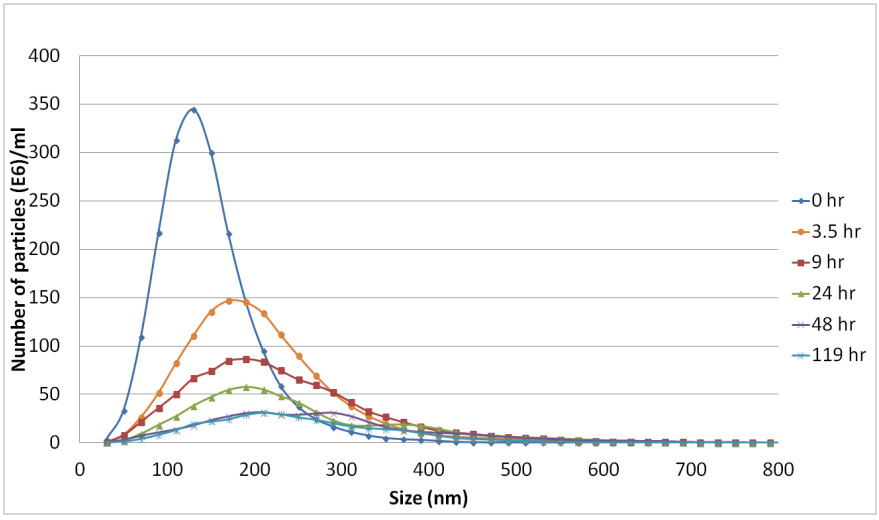
Figure 4.2: Nanosight measurements of PBCA nanoarticles in different pH buffer systems. The concentration for all of them are 1:300 and all of them are carried out in the dialysis-cassette setup. The last measurements (>300 hours/13 days) for pH 7.4 were carried out with a higher gain in order to actually see some particles. The concentration may therefore by accident have been measured to a higer value. The STD is a measure of the particle size distribution width. A large STD means a polydisperse sample, whereas a low STD means a monodisperse sample.



(a) Particle distribution of PBCA 1:500

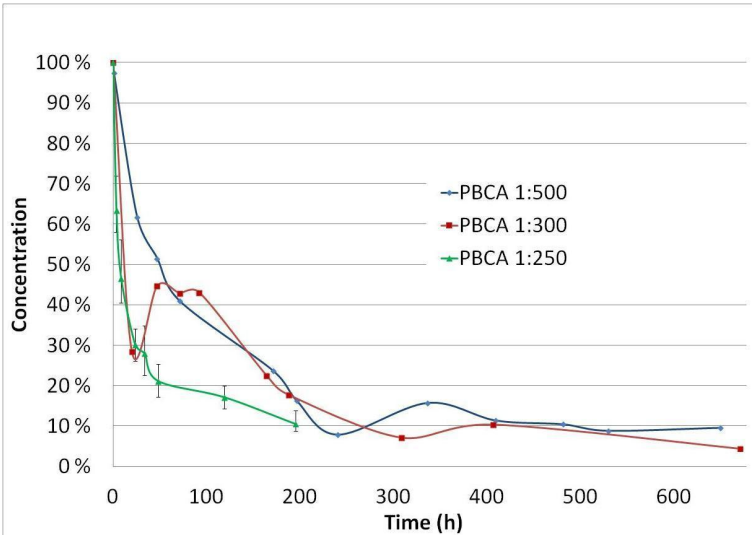


(b) Particle distribution of PBCA 1:300

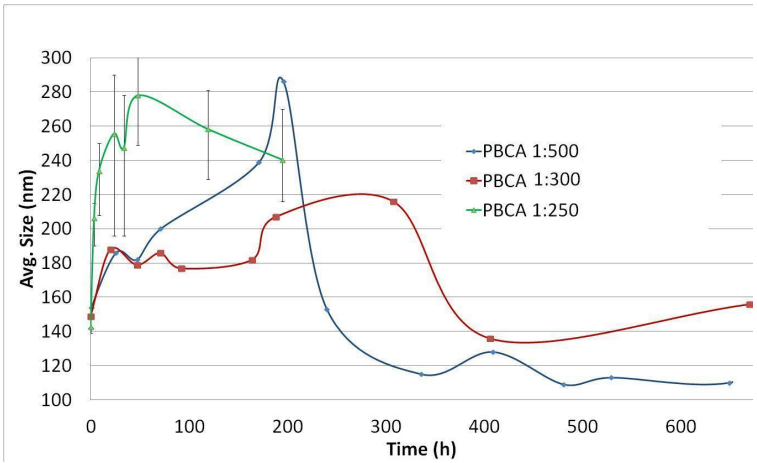


(c) Particle distribution of PBCA 1:250

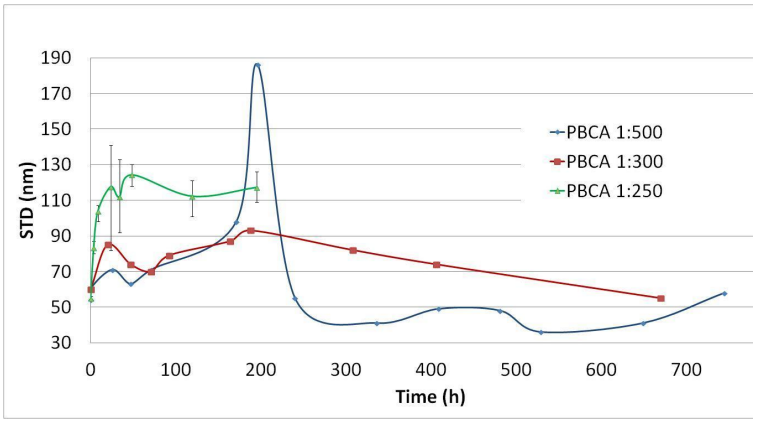
Figure 4.3: Nanosight measurements of PBCA nanoarticles in buffer pH 7.4 with different concentrations. 1:500 and 1:300 are run with the dialysis-cassette setup and there was only one parallel for each of them. For the 1:250 there are 3 paralell samples, and the average value is the foundation for the curve. The two last measurements for PBCA 1:300 and 1:500 were run with a higher gain than the others. Hence, the concentration may be higher than what it is supposed to be. However, the mean size should be right, for more information see section 5.4.1



(a) Concentration decay for different dilutions of PBCA in buffer pH 7.4

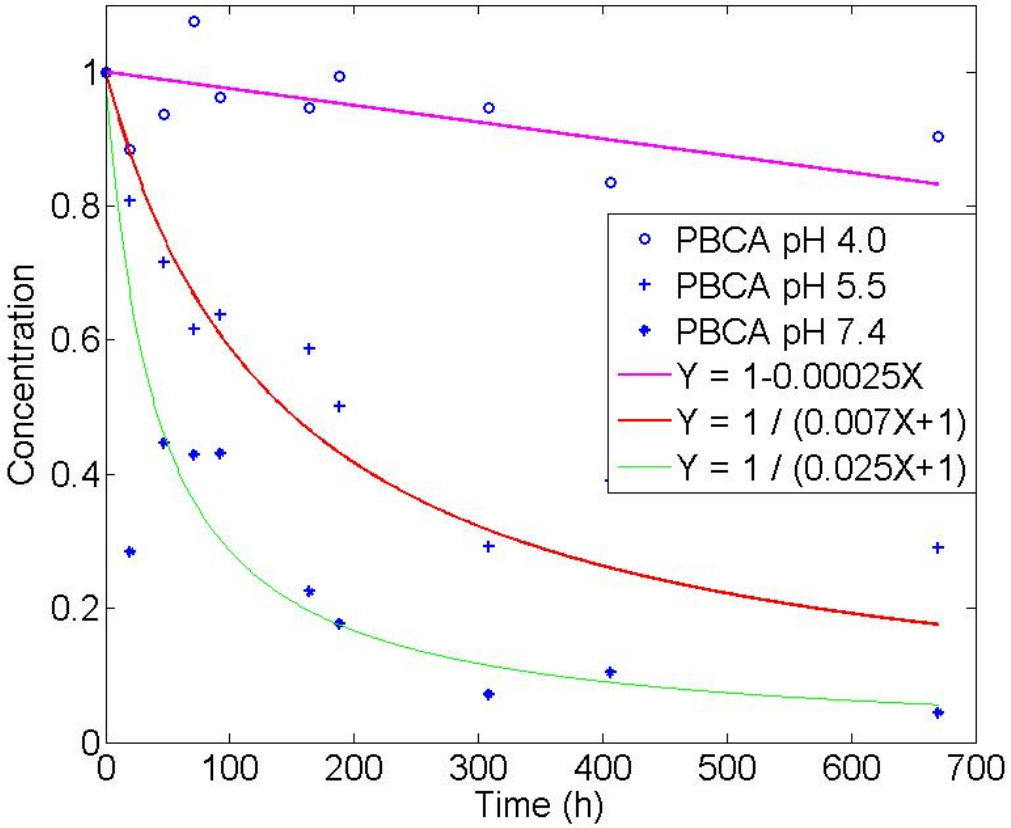


(b) Particle size for different dilutions in buffer pH 7.4. Mark the sudden drop after 200 and 350 hours for 1:500 and 1:300 respectively.

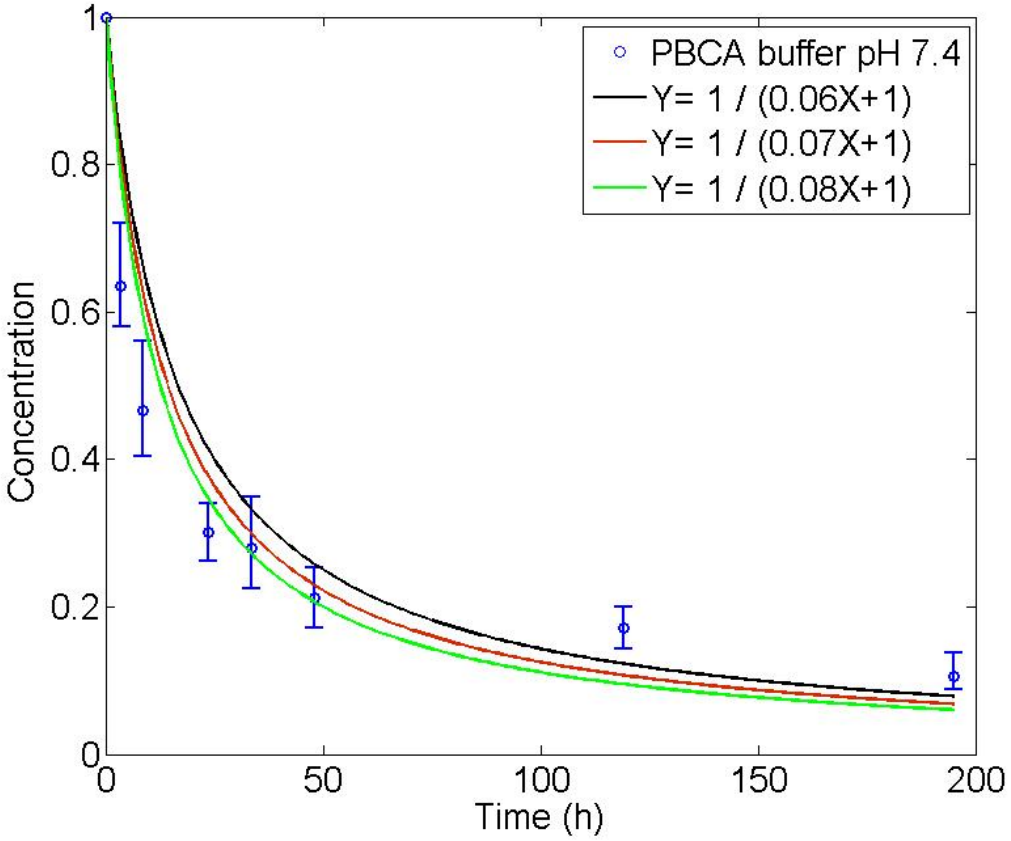


(c) Width of particle distribution of PBCA particles in buffer pH 7.4 with different concentrations

Figure 4.4: Nanosight measurements of PBCA nanoarticles in buffer pH 7.4 with different concentrations. 1:500 and 1:300 are run with the dialysis-cassette setup and there was only one parallel for each of them. For the 1:250 there are 3 paralell samples, and all of them uses the reagent bottle setup. The curve for 1:250 is based on the average value of the three samples. The error bars gives the highest and lowest value of these three values. The STD is a measure of the particle size distribution width. A large STD means a polydisperse sample, whereas a low STD means a monodisperse sample.

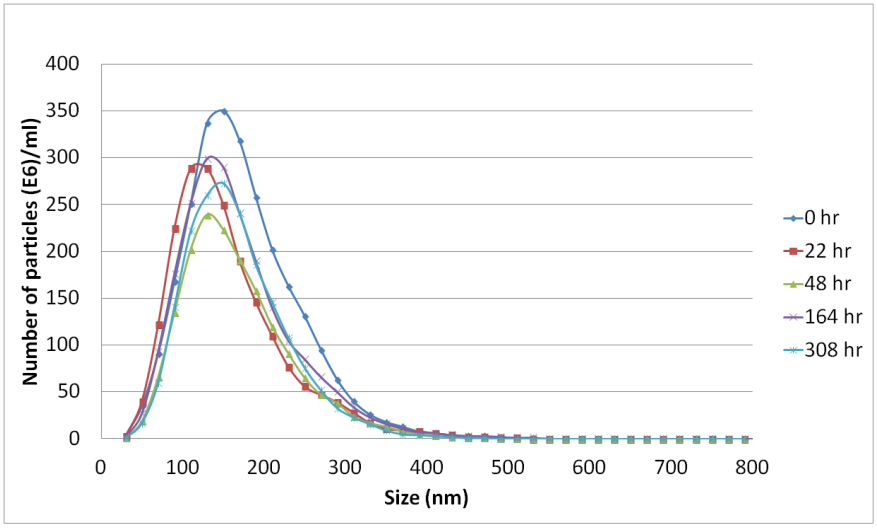


(a) Curve fitting for PBCA 1:300. pH 4.0 follows a linear decay, whereas pH 5.5 and 7.4 fit good with a 1/x-decay. The dialysis casette method is used for this samples, and only one paralell for each

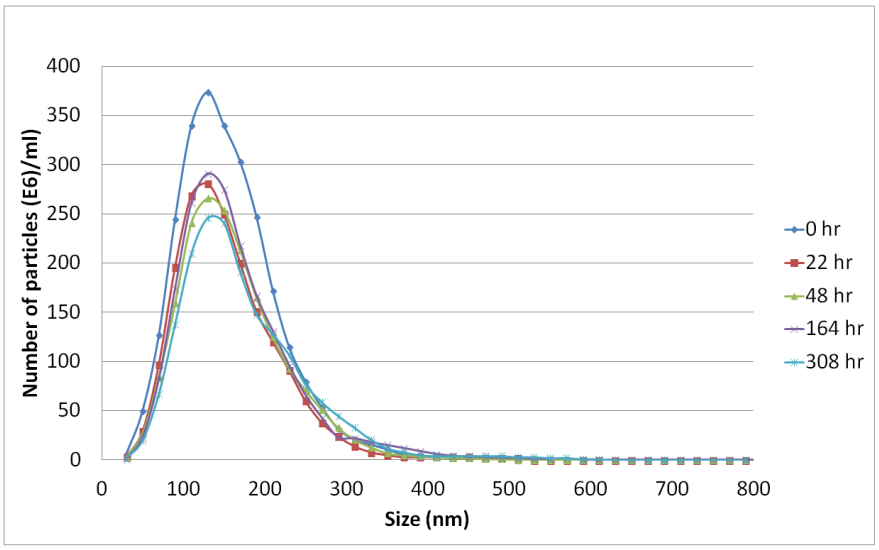


(b) The data points from PBCA 1:250 pH 7.4 is plotted versus different $\frac{1}{x}$-functions, and they seem to overlap relatively good. The reagent bottle method is used for these samples. The data points are obtained from 3 paralell samples.

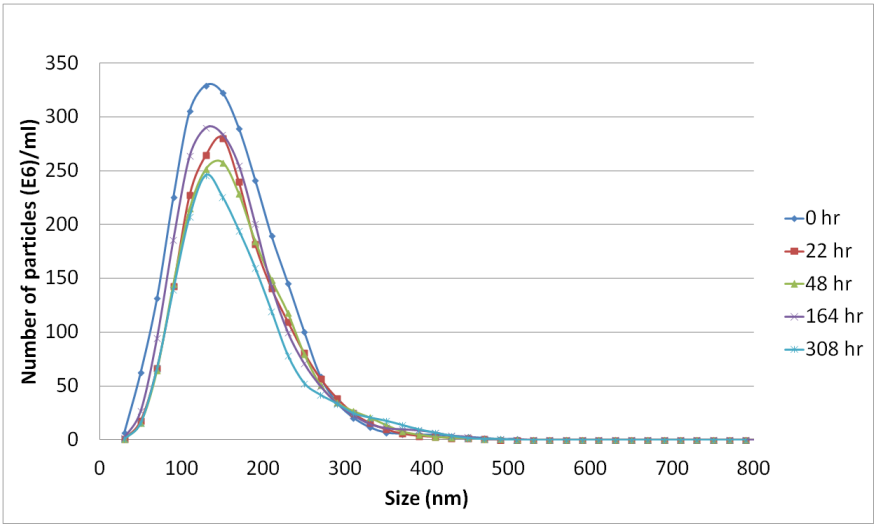
Figure 4.5: Curve fitting in Matlab for PBCA particles in different pHs.



(a) Particle distribution of POCA buffer pH 4.0.

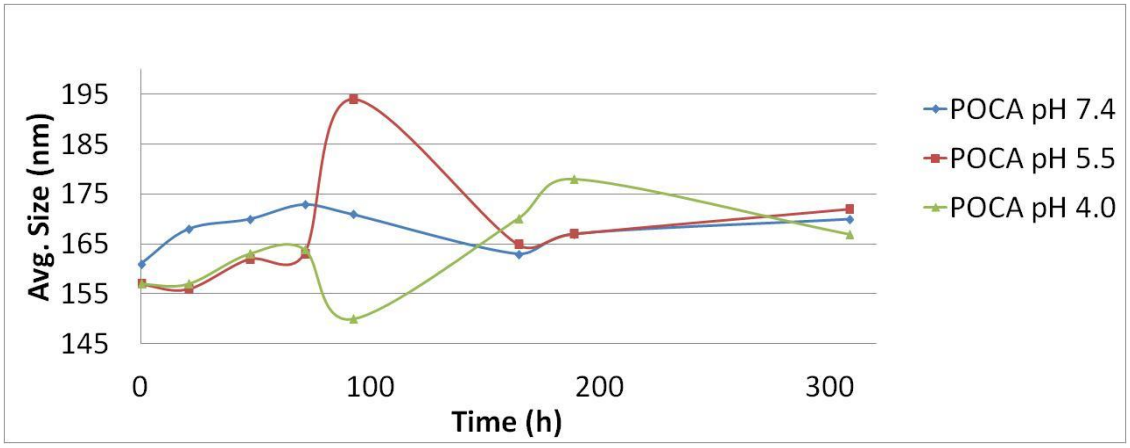


(b) Particle distribution of POCA buffer pH 5.5

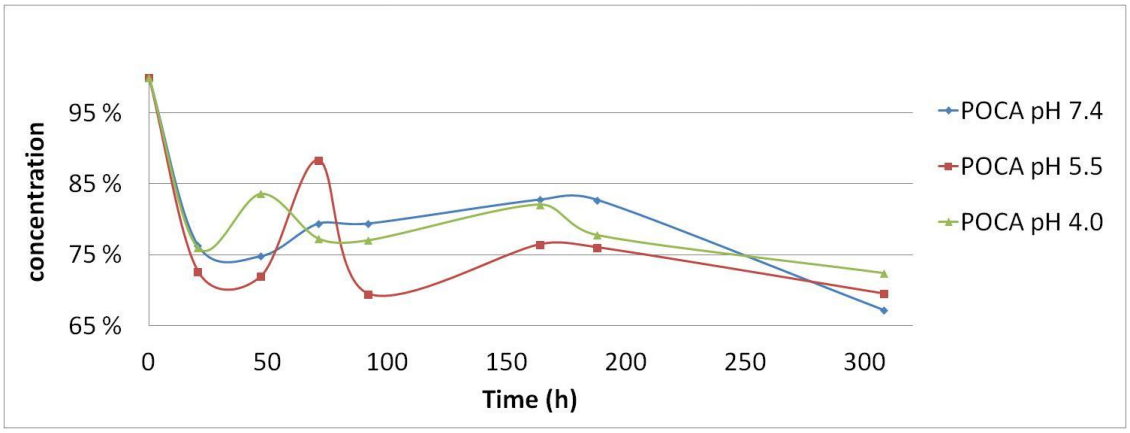


(c) Particle distribution of POCA buffer pH 7.4

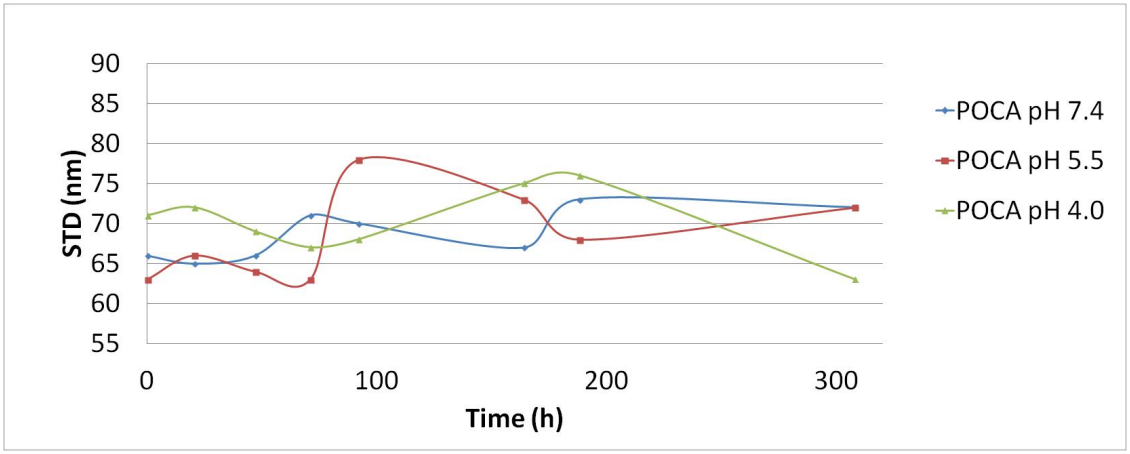
Figure 4.6: Nanosight measurements of POCA in different buffers. All samples are diluted to a 1:300 concentration, and the dialysis-cassette setup is used.



(a) Particle size of POCA nanoparticles in different pHs



(b) Particle concentration of POCA nanoparticles in different pHs.



(c) Width of particle distribution of POCA nanoparticles in different pHs.

Figure 4.7: Nanosight measurements of POCA nanoarticles in different pH buffer systems. The concentration for all of them are 1:300 and all of them are carried out in the dialysis-cassette setup. The STD is a measure of the particle size distribution width. A large STD means a polydisperse sample, whereas a low STD means a monodisperse sample.

4.2 Nanosight - Different mediums with constant pH

To be able to understand more of the degradation processes, degradation in different mediums were carried out. The different mediums are buffer pH 7.4 with and without esterase, cell medium and blood serum.

4.2.1 PBCA in different mediums

The concentration, mean size and standard deviation for PBCA particles in the four different mediums are given in Figure 4.9. The result of PBCA particles in buffer pH 7.4 is already described in section 4.1.1, but is plotted again in Figure 4.10a in order to better compare it with the other mediums.

PBCA in blood serum

The particles in blood serum (Figure 4.10b) were the only particles that actually had a steady mean size reduction . The initial mean size for PBCA particels is 235 nm, whereas the size has decreased to 170 nm after 100 hours. The decrease was most dominant the first hours. The concentration of PBCA particles followed approximately the same pattern as for buffer pH 7.4, in which the decrease was really fast the first 10 hours, followed by a more gradual decrease before it seems to stabilize. After 100 hours the concentration is decreased to 30%. Even though the concentration decays were similiar in buffer pH 7.4 and blood serum for PBCA particles, their behaviour in these mediums was still different. The STD for instance. In buffer pH 7.4 it started low and increased, whereas STD in blood serum started high and did reduce.

PBCA in cell medium

Cell medium was the most stable environment for PBCA particles (Figure 4.11a). The concentration decayed less than for the other mediums and it stabilized around 80%. The initial size was 172 nm, which was higher than in buffer pH 7.4, but smaller than in blood serum. After 3.5 hours the whole particle distribution has shifted towards a slightly higher size. Over time the mean size and the STD did not follow a clear pattern, instead they fluctuated.

PBCA in buffer pH 7.4 with esterase

The esterase-samples (Figure 4.11b) behaved differently from all the other samples. They sedimented clearly within a few hours (Figure 4.8). The concentration was relatively stable the first 15 hours, then it suddenly increased up to a level which was 60% higher than the original value. After 24 hours the samples of esterase were finally installed in a rotating device to inhibit sedimentation. First then a significant change in the concentration was seen. The concentration decreased from 160% to 40% within the next 24 hours.

The measurement after 3.5 hours looks similar to the one for buffer pH 7.4 without esterase, except that less particles have disappeared. The initial size for PBCA was quite high 221 nm and the increase continued almost linearly the next 100 hours to 347 nm, which is the higest measured mean size for all samples.

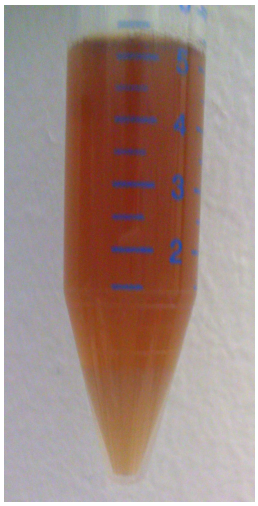


Figure 4.8: Sedimentation of particles in the buffer-esterase sample

4.2.2 POCA in different mediums

The concentration, mean size and standard deviation for POCA for all the different mediums are given in Figure 4.12.

POCA in buffer pH 7.4

For POCA particles (1:250) in buffer pH 7.4 (Figure 4.13a) not much happened. A slightly mean size increase was seen during 5 days of degradation, whereas the concentration did not change much. A small increase was actually observed.

POCA in blood serum

The mean size and STD of POCA in blood serum, was just as PBCA, initially high and decreased with time (Figure 4.13b). However, in contrast to PBCA, the POCA concentration was maintained high. The POCA concentration reduced slowly to ca. 80% within 100 hours. Although this was not much, this was the only medium in reagent bottles with a clear decrease in particle concentration.

POCA in cell medium

In cell medium, the POCA particles had an initial mean size and STD larger than in buffer pH 7.4, but smaller than in blood serum (Figure 4.14a). However, neither the concentration nor the STD changed much during 5 days of degradation.

POCA in buffer pH 7.4 with esterase

Just as the PBCA samples, the POCA samples were affected by sedimentation, and the concentration became very high. However, after rotation was started a significant concentration decay was seen, but the concentration did not decrease below the initial concentration. The STD of the particle size distribution increased to a very high level, the highest for all POCA particle samples.

4.2.3 Initial size of particles varies

The initial size of PBCA and POCA particles varied between the different mediums due to protein adsorption. These can be found in Table 4.1. The initial size was largest in blood

serum for both PBCA and POCA. The size of the adsorbed layer for POCA particles was slightly smaller than for PBCA particles in all mediums.

Table 4.1: Initial size of PBCA and POCA particles in different mediums

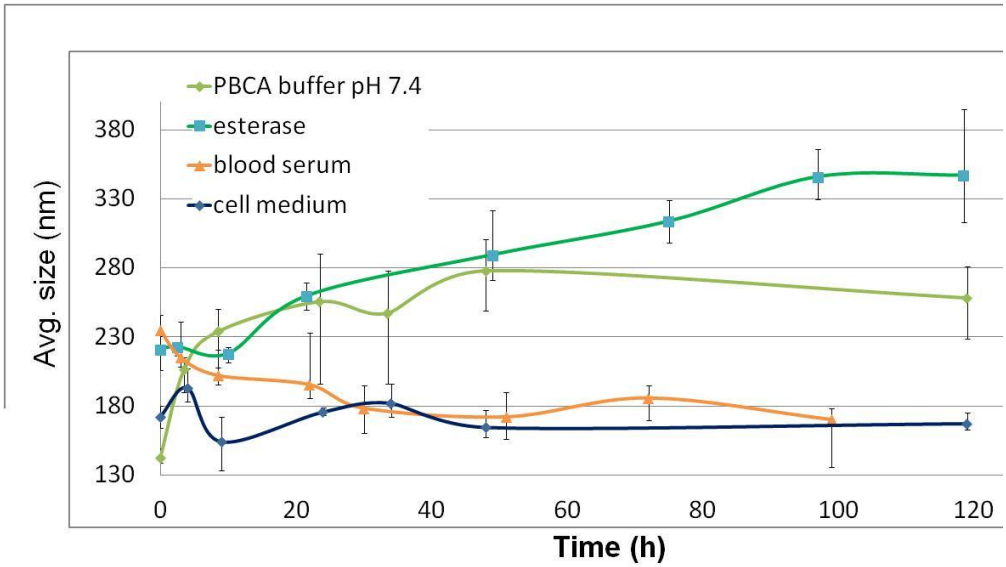
Type	**Medium**	**Initial size**[1]	**Size of protein layer**
PBCA[2]	Buffer pH 7.4 without esterase	145 nm	0 nm
PBCA	Cell medium	172 nm	13.5 nm
PBCA	Buffer pH 7.4 with esterase	221 nm	38 nm
PBCA	Blood serum	235 nm	45 nm
POCA[3]	Buffer pH 7.4 without esterase	155 nm	0 nm
POCA	Cell medium	180 nm	12.5 nm
POCA	Buffer pH 7.4 with esterase	206 nm	25.5 nm
POCA	Blood serum	225 nm	35 nm

[1] The initial size of the particles are calculated from the initial size of 3 paralell samples.
[2] All PBCA particles come from batch YM 66
[3] All POCA particles come from batch YM 64

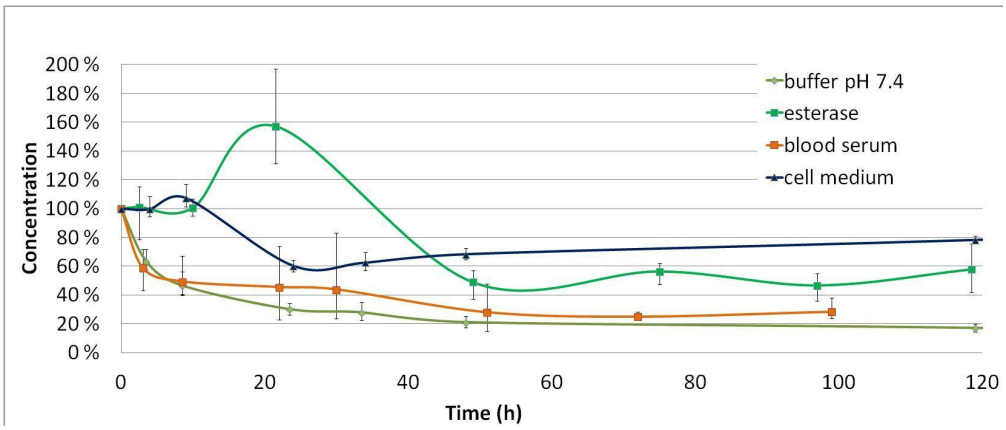
4.3 Electron microscopy for detection of degradation

A few images were taken by a scanning electron microscope in order to hopefully see some effects from degradation. PBCA images are shown in Figure 4.15 and POCA imges in Figure 4.16. Great changes can be seen in the images of PBCA. Initially the particles were spherical and the contact area between neighbouring particles were small. In the image taken after 5 days, particles did not anymore have this smooth surface. At this point it is hard to identify each single particle, because they have kind of melted together and the contact area between neighbouring particles has definitely increased. In the final image taken after 11 days, no particles could be seen.

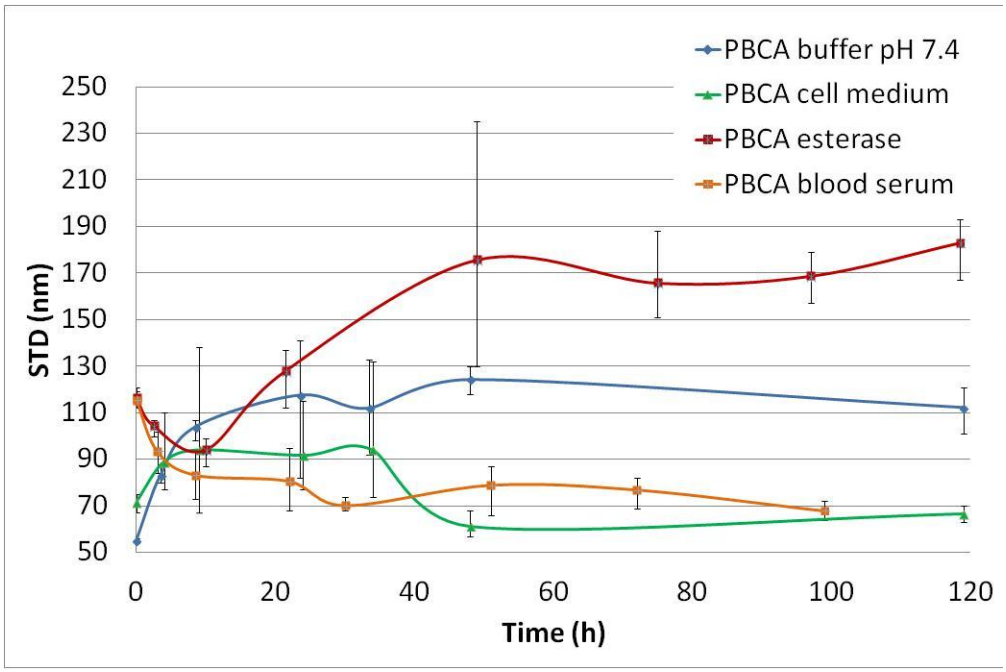
For POCA particles no significant change could be seen between the three images taken after 0 hour, 5 days and 11 days.



(a) Size of PBCA nanoparticles

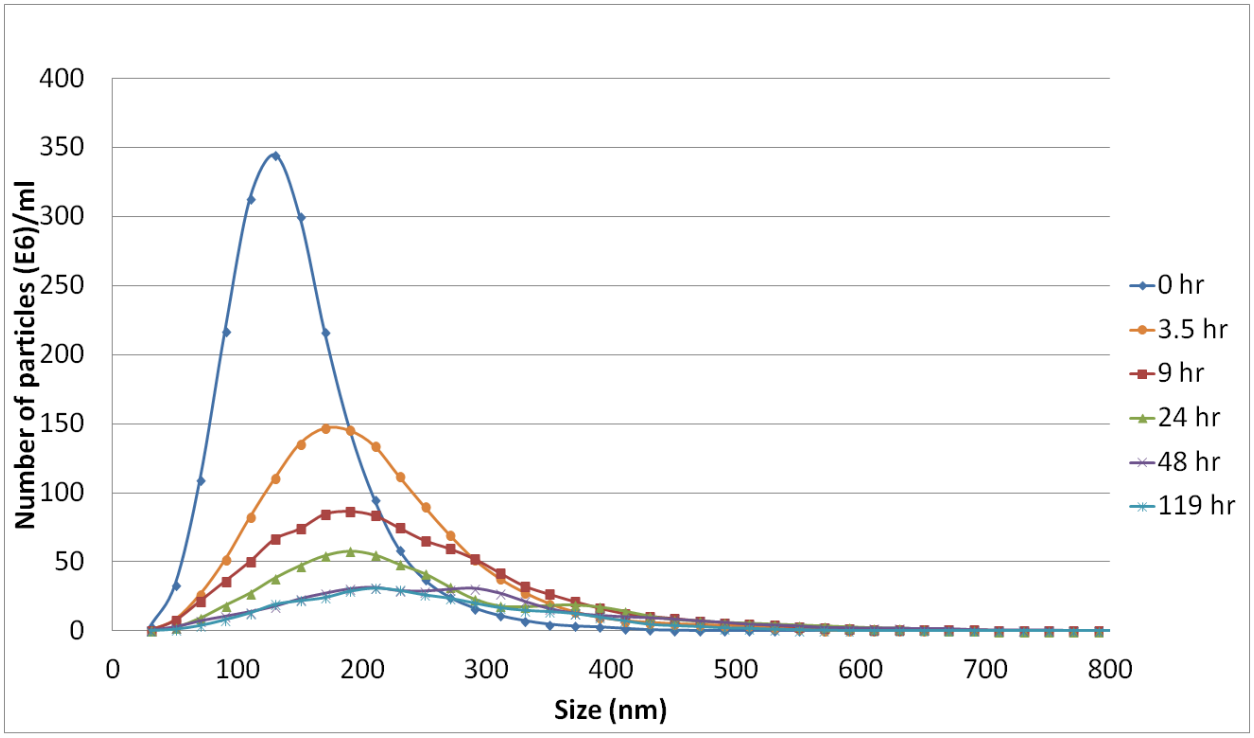


(b) Particle concentration of PBCA nanoparticles

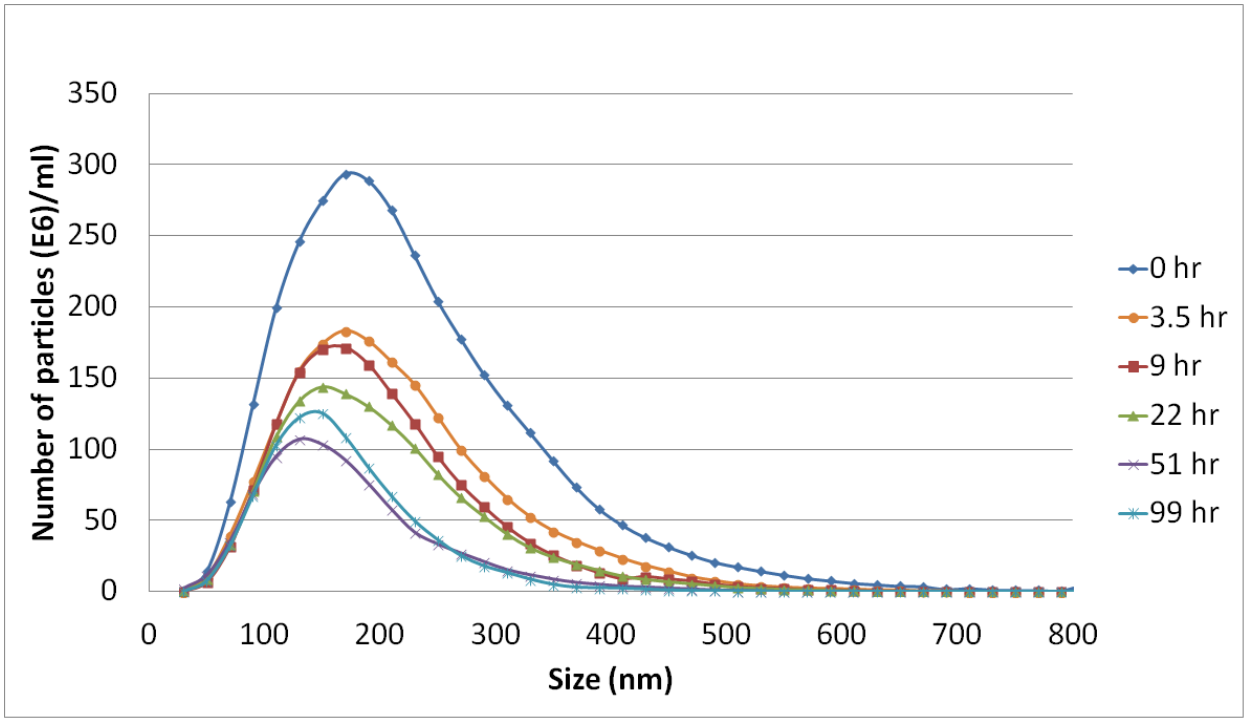


(c) The width of the particle distributions for PBCA particles in different mediums

Figure 4.9: The particle size and concentration of PBCA 1:250 for buffer pH 7.4, esterase, blood serum and cell medium. Each curve is obtained from the average of 3 equal samples. The reagent bottle-method is used for these samples. The STD is a measure of the particle size distribution width. A large STD means a polydisperse sample, whereas a low STD means a monodisperse sample.

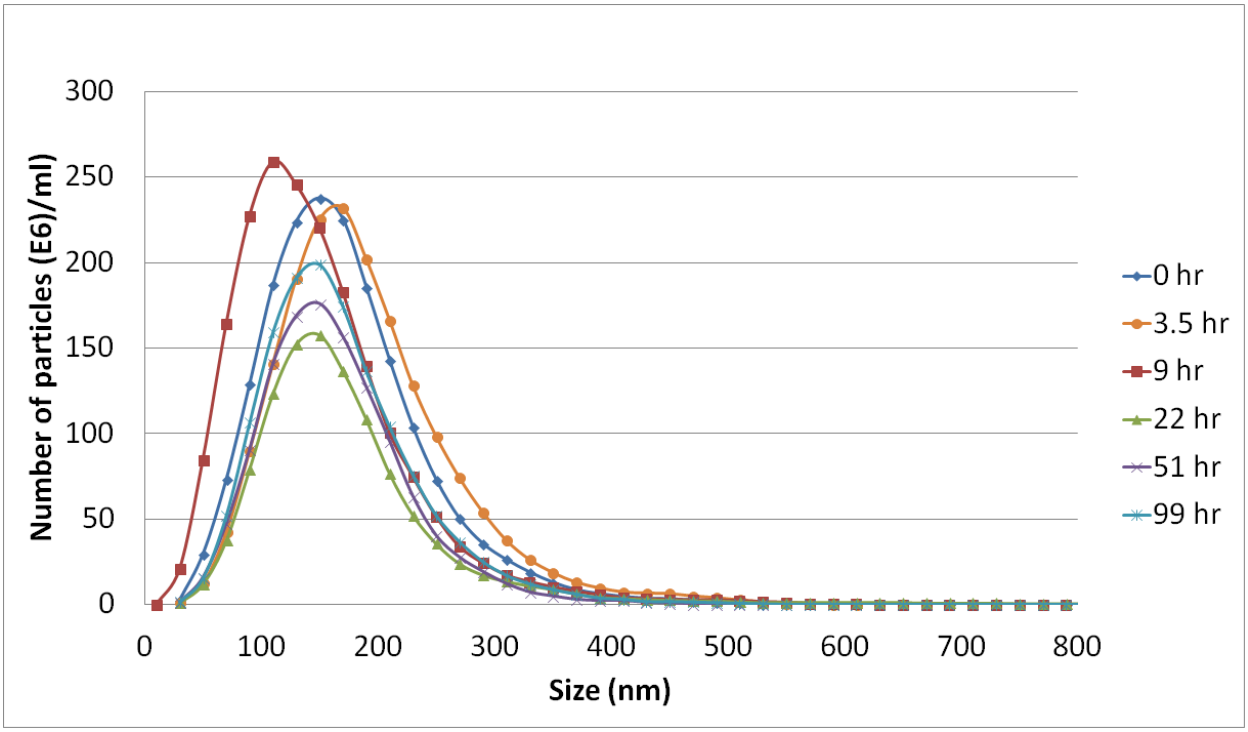


(a) Particle size distribution of PBCA nanoparticles in buffer pH 7.4

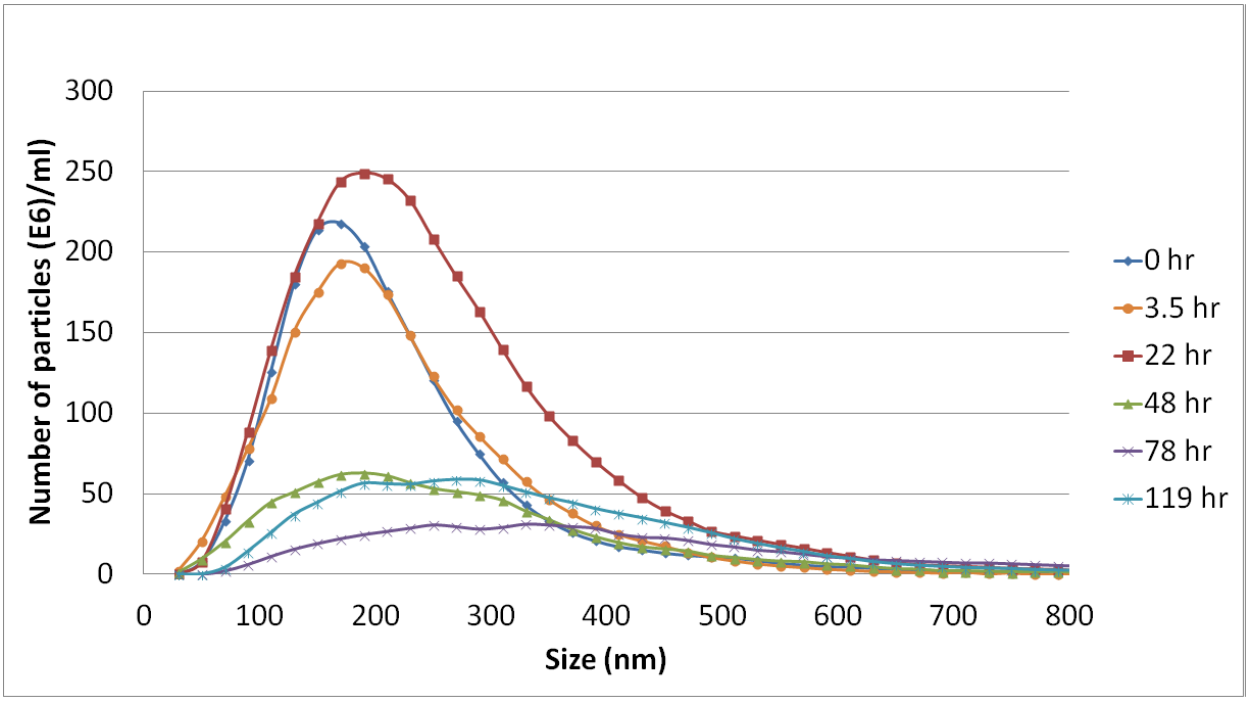


(b) Particle size distribution of PBCA nanoparticles in blood serum

Figure 4.10: Nanosight measurements of PBCA nanoarticles in buffer pH 7.4 and blood serum. The dilution is 1:250. The reagent bottle-method is used. The curves are obtained from the average of 3 paralell samples.

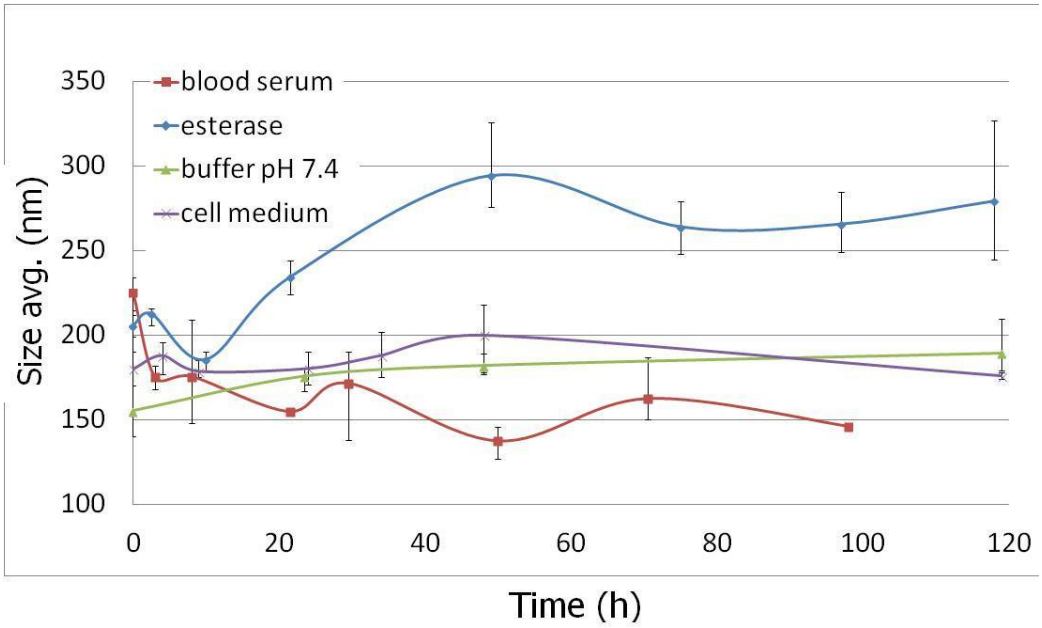


(a) Particle size distribution of PBCA nanoparticles in cell medium

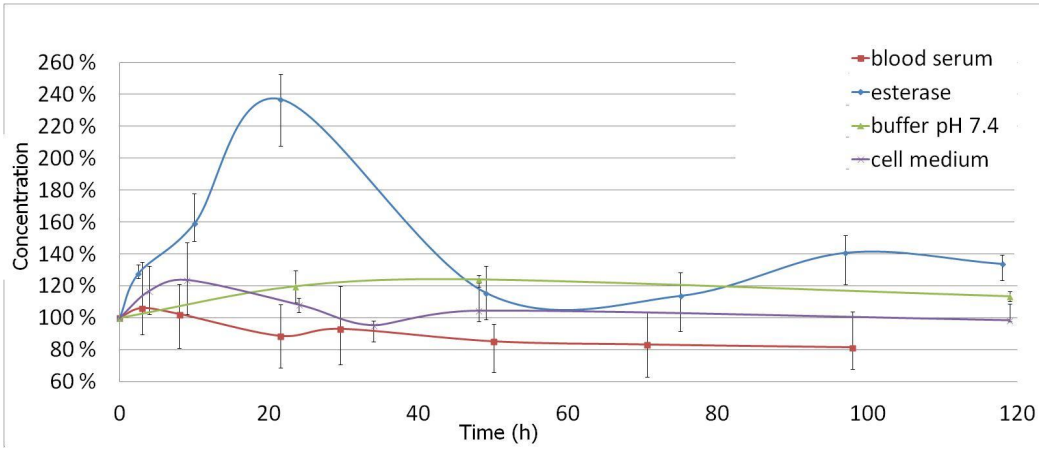


(b) Particle size distribution of PBCA nanoparticles in buffer pH 7.4 with esterase

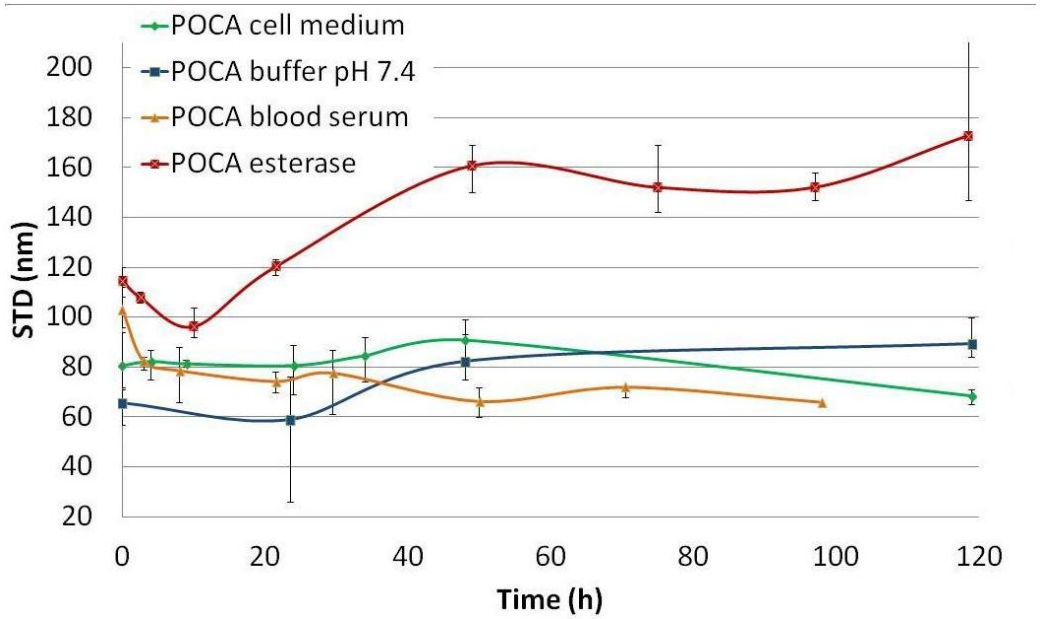
Figure 4.11: Nanosight measurements of PBCA nanoarticles in cell medium and buffer pH 7.4 with esterase. The dilution is 1:250. The reagent bottle-method is used. The curves are obtained from the average of 3 paralell samples. The particles in esterase swelled quickly (Figure 4.8) and the samples were after 24 hours placed in a rotating device. Thereafter no sedimentation were present.



(a) Size of POCA nanoparticles in different mediums.

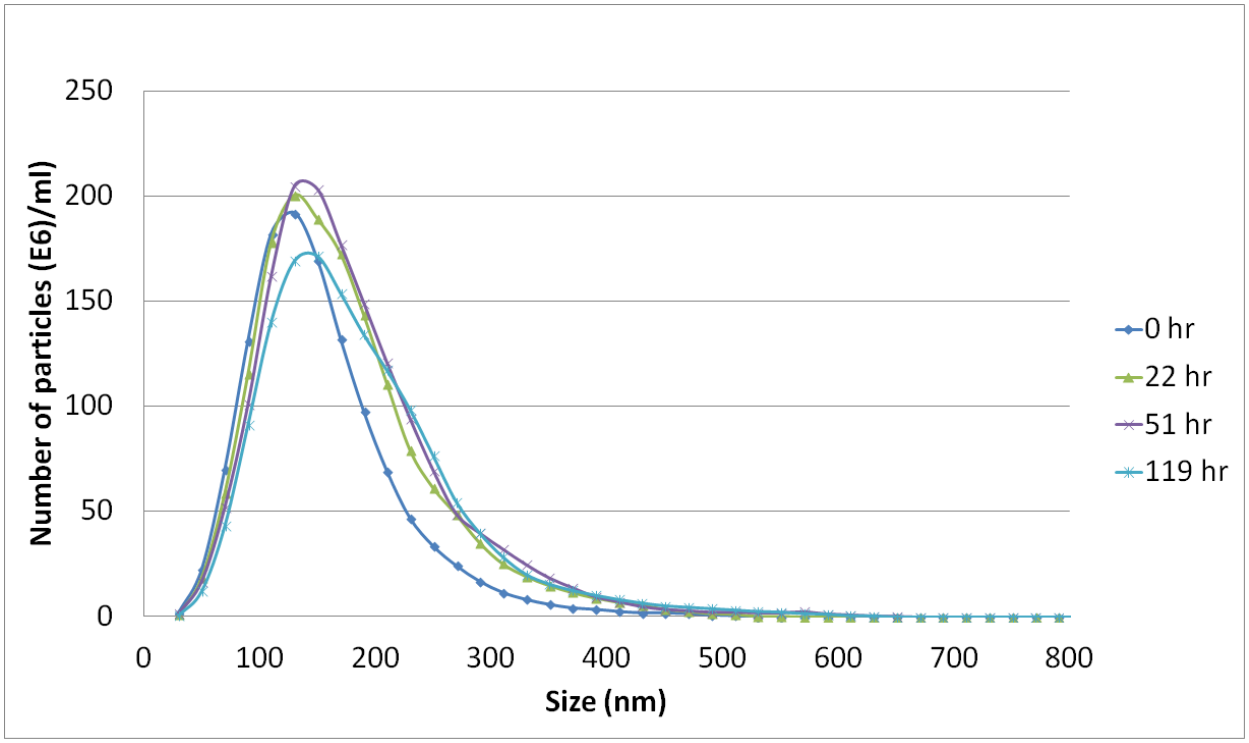


(b) Particle concentration of POCA nanoparticles in different mediums.

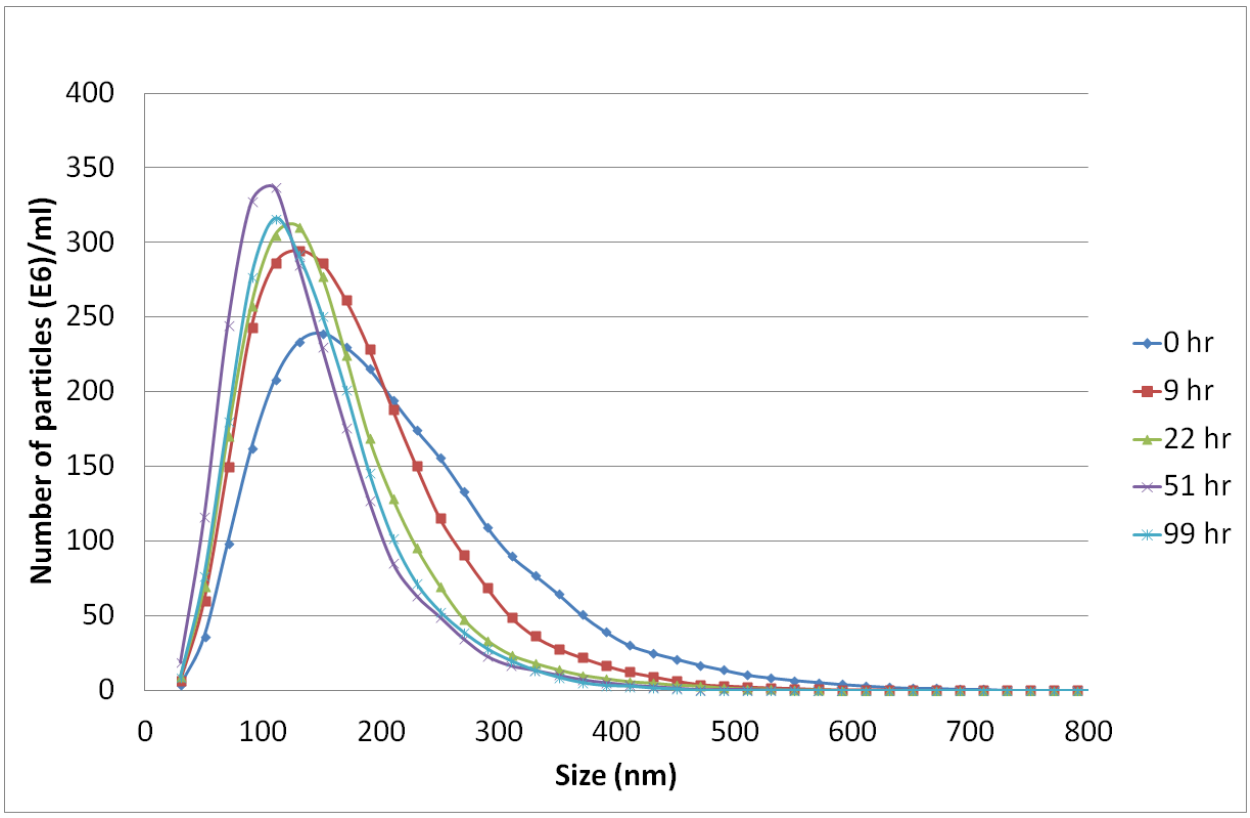


(c) The width of the particle distributions for PBCA particles in different mediums

Figure 4.12: The particle size and concentration of POCA 1:250 for buffer pH 7.4, esterase, blood serum and cell medium. Each curve is obtained from the average of 3 equal samples. The reagent bottle-method is used for these samples. The STD is a measure of the particle size distribution width. A large STD means a polydisperse sample, whereas a low STD means a monodisperse sample.

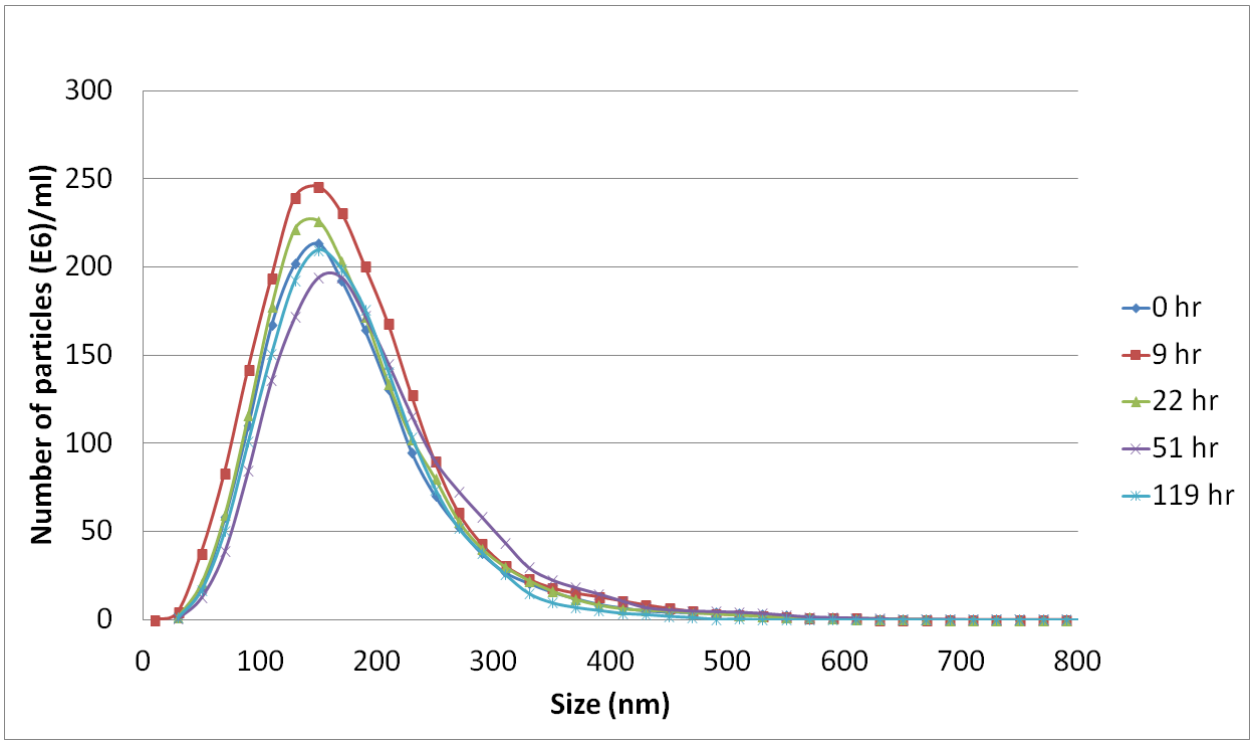


(a) Particle size distribution of POCA nanoparticles in buffer pH 7.4.

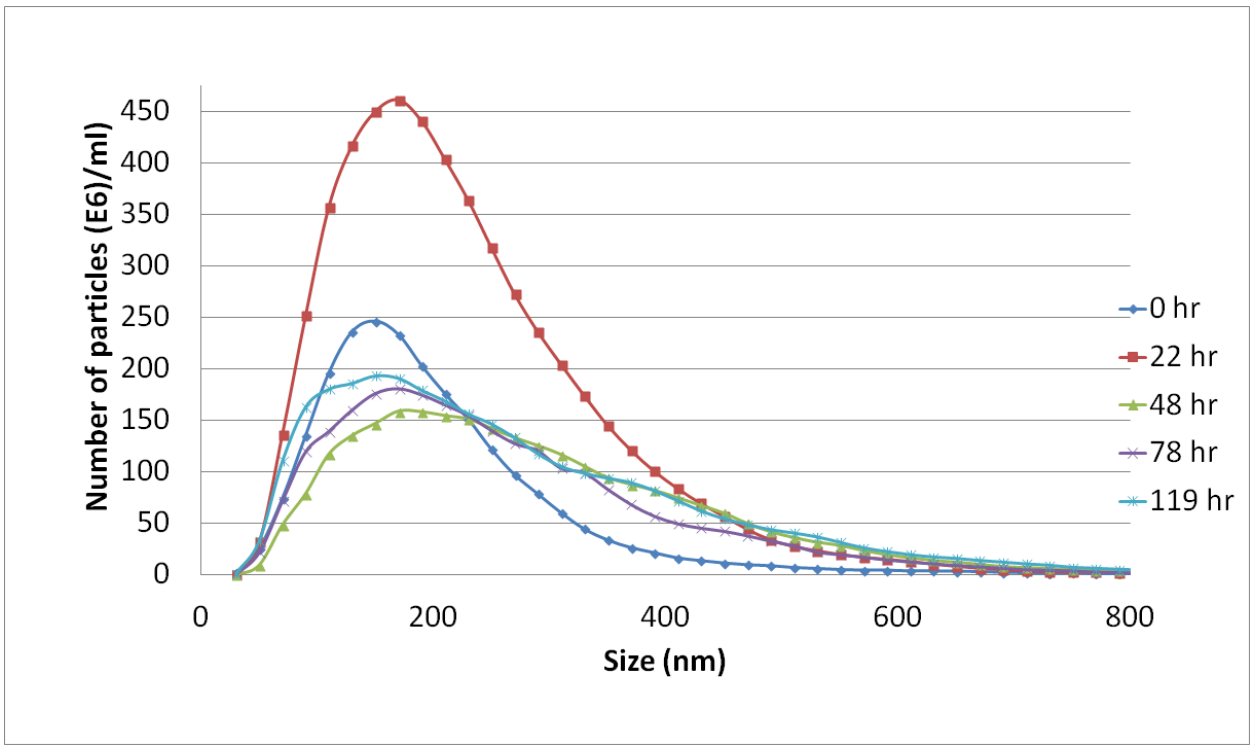


(b) Particle size distribution of POCA nanoparticles in blood serum

Figure 4.13: Nanosight measurements of POCA nanoarticles in buffer pH 7.4 and blood serum. The dilution is 1:250. The reagent bottle-method is used. The curves are obtained from the average of 3 paralell samples.

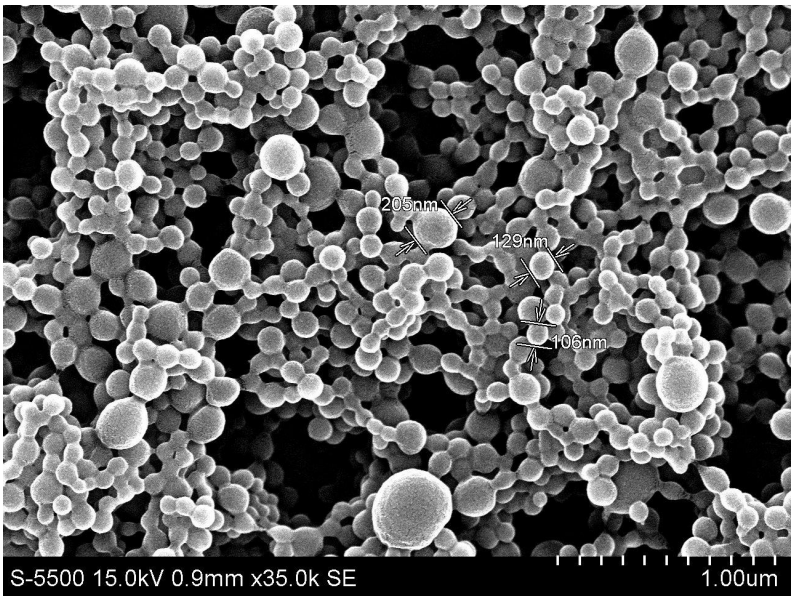


(a) Particle size distribution of POCA nanoparticles in cell medium

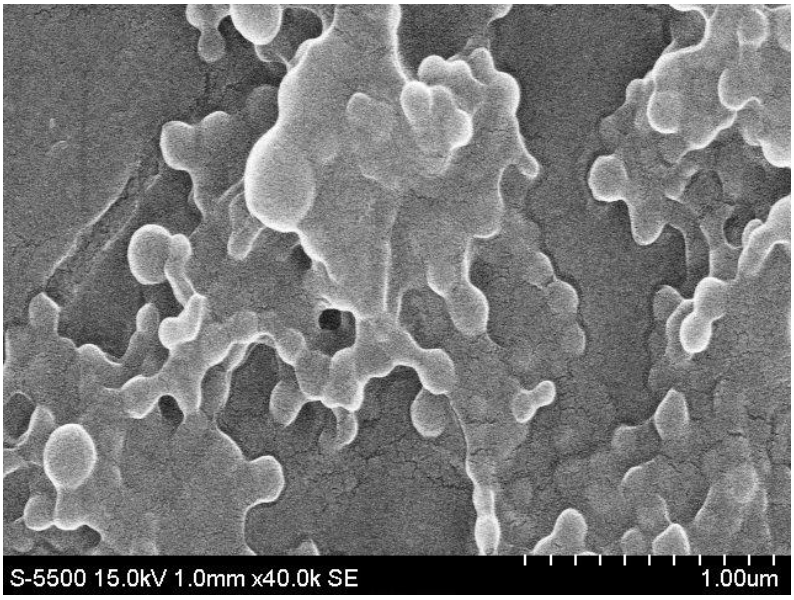


(b) Particle size distribution of POCA nanoparticles in buffer pH 7.4 with esterase

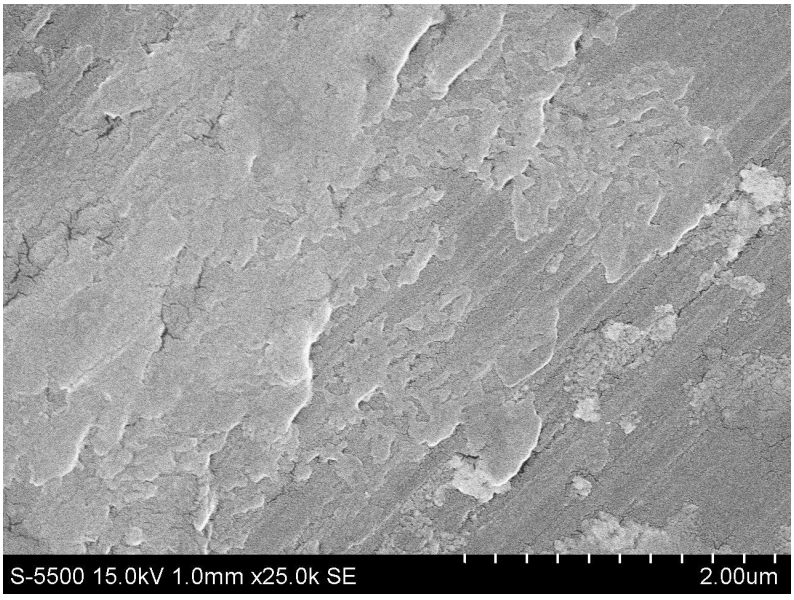
Figure 4.14: Nanosight measurements of POCA nanoarticles in cell medium and in buffer pH 7.4 with esterase. The dilution is 1:250. The reagent bottle-method is used. The curves are obtained from the average of 3 paralell samples.



(a) PBCA NP day 1

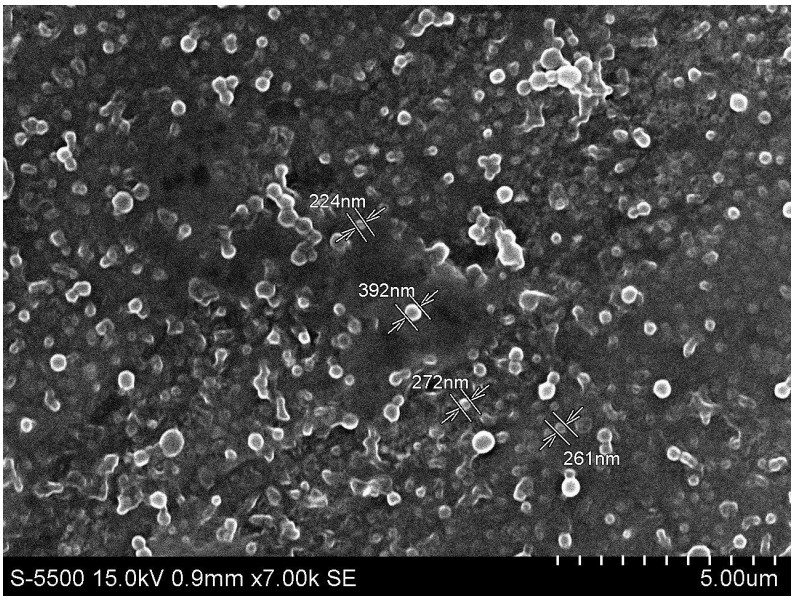


(b) PBCA NP day 5

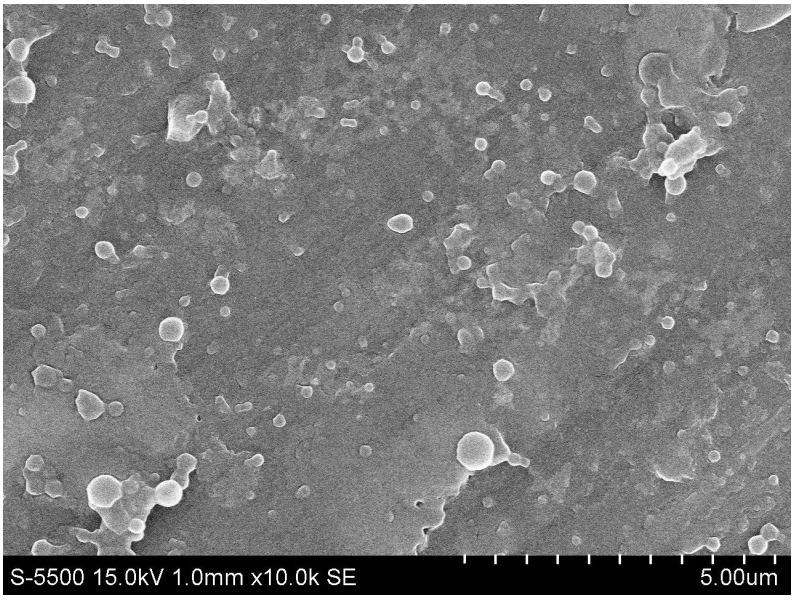


(c) PBCA NP day 11

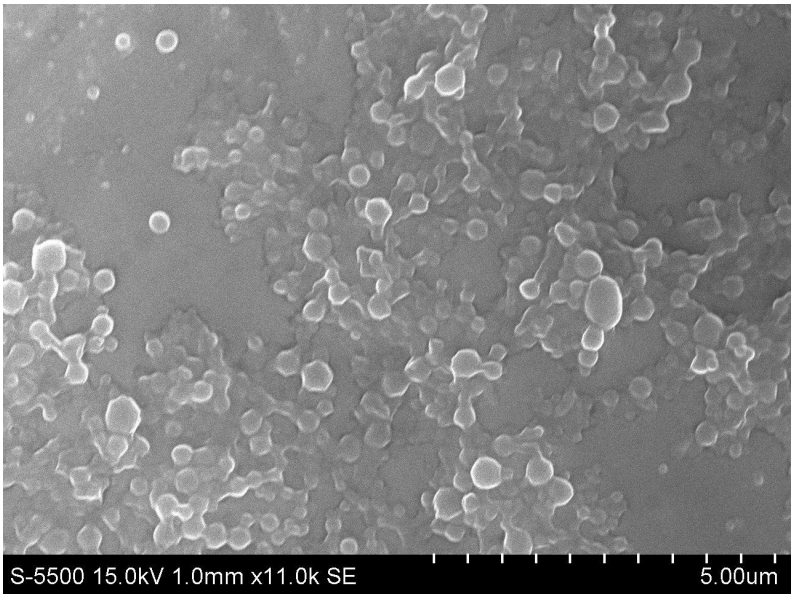
Figure 4.15: Scanning electron microscope images of PBCA nanoparticles held in a buffered solution at pH 7.4. The particles are coated with 10 nm of gold in order to reduce noise.



(a) POCA NP day 1



(b) POCA NP day 8



(c) POCA NP day 11

Figure 4.16: Scanning electron microscope images of POCA nanoparticles held in a buffered solution at pH 7.4. The particles are coated with 10 nm of gold in order to reduce noise.

4.4 Gas Chromatography

Gas Chromatography measurements were carried out on PBCA and POCA particles in buffer pH 7.4 in order to track degradation based on quanitification of the degradation products butanol and octanol, respectively. The different concentrations for PBCA were chosen to see if this could affect the degradation. Tests with butanol, pentanol and octanol revealed that the retention times were 7.51 minutes for butanol, 9.94 minutes for pentanol and 15.88 minutes for octanol.

The maximum amount of butanol and octanol, which is the degradation products of PBCA and POCA, respectively, was found by degrading PBCA and POCA in a 0.05M NaOH-solution for 1 week. The real amount of butanol and octanol was calculated based on the known amount of pentanol present. The maximum mass of butanol present was calculated to be $2.7 \cdot 10^{-6}\, mole$, whereas the maximum mass of octanol was calculated to be $2.5 \cdot 10^{-6}\, mole$. (see Appendix Section 7.3 for calculations). This is lower than the calculated theoretical value (section 2.7.3), which is $7.3 \cdot 10^{-6}\, mole$ for butanol and $4.0 \cdot 10^{-6}\, mole$ for octanol.

POCA particles 1:250 were degraded in buffer pH 7.4 for 44 hours. No octanol was detected. For PBCA, degradation of two series with different concentrations(1:25 and 1:250) were carried out. The results can be seen in Figure 4.17. There is a clear difference between the two curves. Both of them seem to be quite linear, but with a different slope. The 1:250 sample has a butanol yield of 25% after 40 hours, whereas 1:25 has just a few percents.

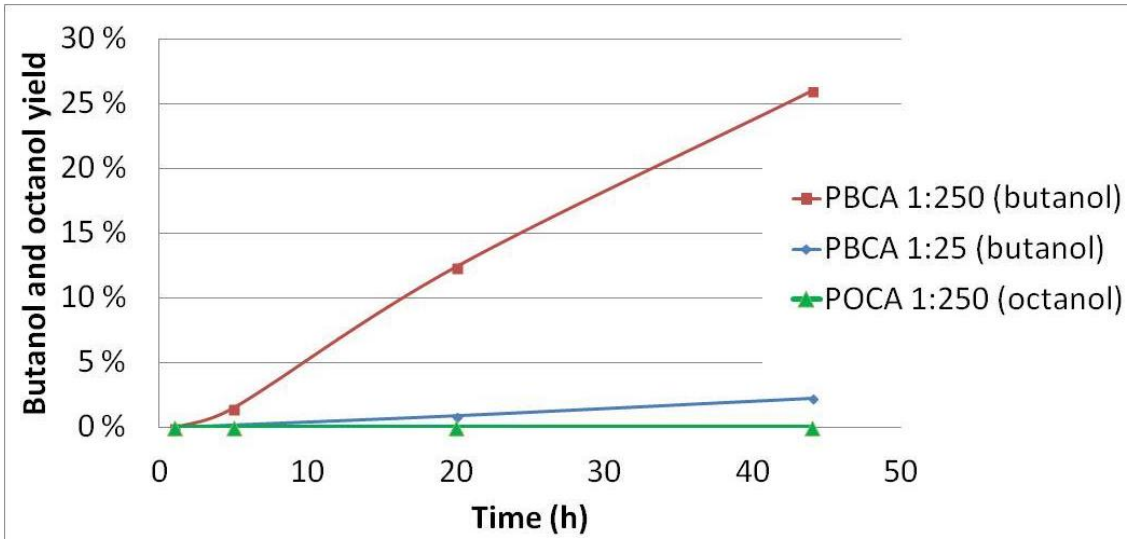


Figure 4.17: Gas Chromatography is used to quantify butanol and octanol, which is the main degradation products of PBCA and POCA nanoparticles, respectively. Two PBCA samples with different dilutions (1:25 and 1:250) were tested, and one POCA sample with dilution 1:25. The yield is calculated based on similar samples that were fully degraded.

4.5 Zetapotential results

4.5.1 Size and PDI measurements

The size and the polydiserpsity index(PDI) were obtained for the PBCA particles in buffer pH 7.4. In Figure 4.18 the results from the Zetasizer are compared with the results from the Nanosight. Two paralell series were run in the Zetasizer, these vary much more than expected, and are not reproduceable. Three paralells were run in Nanosight, and these results are reproduceable. The curves from the Zetasizer have additionally a very high PDI, which is a sign that the results not are reliable.

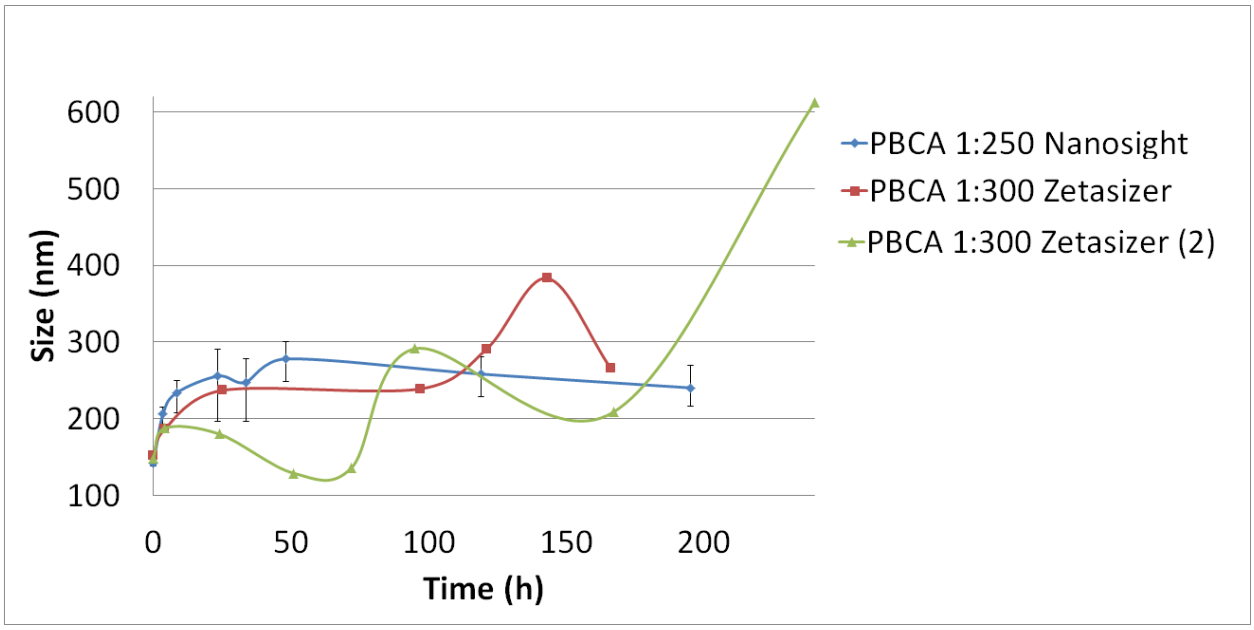
4.5.2 Zetapotential measurements

The zetapotential was measured for PBCA particles with a Zetasizer instrument. The purpose was to see if the zetapotential changed during degradation. The zetapotential was tested with several time intervals, but the measurements were not reliable because of increasing polydiserpsity. However, an interesting observation was made. The zetapotential varied namely with different concentrations. For all measurements done the 1:50 was significantly lower ($ca. -30mV$) than for 1:300 ($ca. -17mV$). A simple test was therefore carried out, in which the zeta potential was measured in different dilutions (1:50, 1:250, 1:500, 1:750, 1:1000 and 1:5000). The results can be found in Table 4.2, and show a decrease in the negative potential (getting closer to zero) with increasing dilution. However, one reaches finally a dilution, in which the zetapotential no longer changes with further dilution.

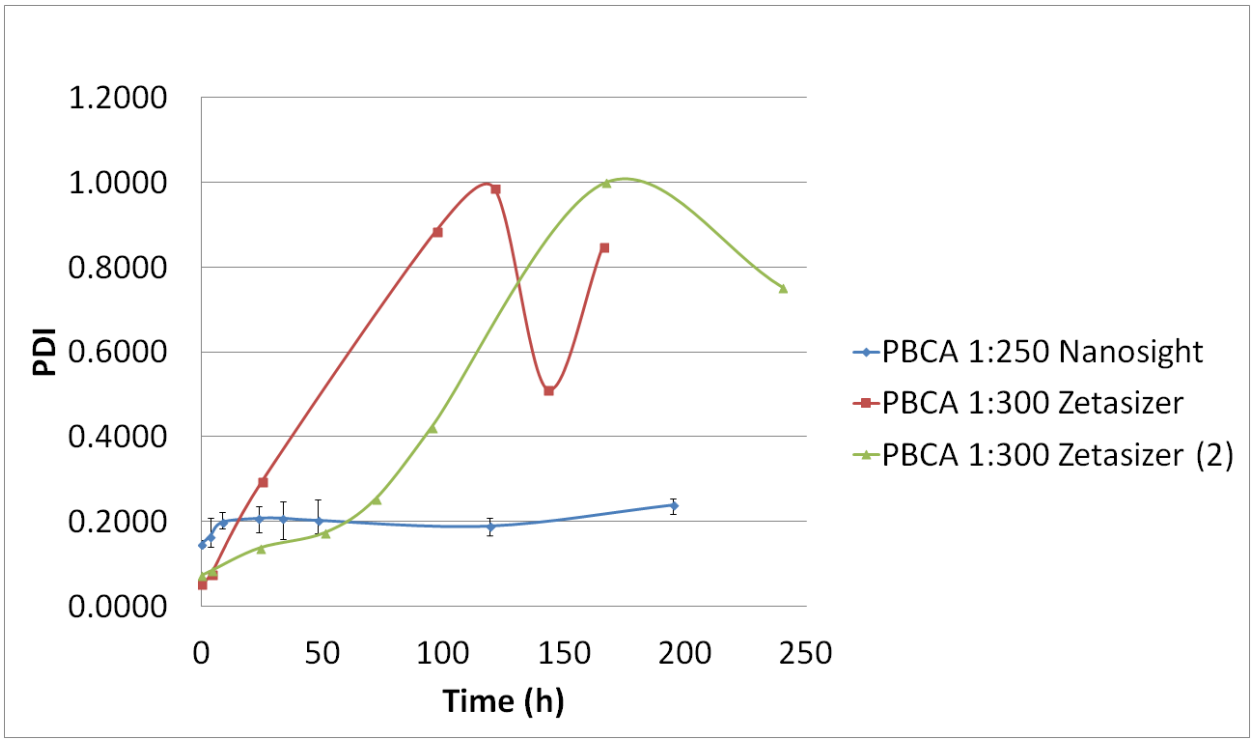
Table 4.2: Zeta-potential vs concentration

Buffer	Dilution	Zetapotential
0.01M Phosphate buffer pH 7.4	1:50	-28.5 mV
0.01M Phosphate buffer pH 7.4	1:250	-22.1 mV
0.01M Phosphate buffer pH 7.4	1:500	-17.9 mV
0.01M Phosphate buffer pH 7.4	1:750	-15.2 mV
0.01M Phosphate buffer pH 7.4	1:1000[1]	-19.6 mV
0.01M Phosphate buffer pH 7.4	1:5000	-15.8 mV

[1] The measurement gave a distribution of zetapotentials, but average was -19.6 mV.



(a) The Figure compare the mean size obtained from the Zetasizer with the mean size obtained from the Nanosight.



(b) The Figure compare the polydiserpity index(PDI) from the Zetasizer with the PDI calculated from the Nanosight. The Nanosight gives actually the particle distribution in standard deviation. But the standard deviation is transformed to a corresponding PDI-value through Equation (2.6)

Figure 4.18: The Figure compare the mean size and the PDI obtained on similar particle systems from the Zetasizer and the Nanosight. Two paralell series are run on the Zeteasizer, and three paralell series are run on the Nanosight, described by the error bars. The error bars show the highest and the lowest value within the paralells.

4.6 Optimizing parameters for degradation setup

Several stability-experiments were carried out in the beginning of the project in order to find the right setup parameters. The results of the tests are listed below in Table 4.3 Summarized, only particles in buffer pH 7.4 and in water were sufficiently stabile with rotation. Therefore rotation was not included in the final setup. However, particles were primarily stabile in all solution as long as they were not rotated.

Table 4.3: Stability experimentes and optimization of parameters

Sample	Medium	Dilution	Rotation	Initially stabile	Commentar
PBCA[1]	Phosphate buffer pH 7.4	1:50	17 rpm	Yes	Aggregated after 3 hours[2]
PBCA	Phosphate buffer pH 7.4	1:300	17 rpm	Yes	No aggregation seen
PBCA	Acetate buffer pH 5.5	1:50	17 rpm	Yes	Aggregated after 3 hours
PBCA	Acetate buffer pH 5.5	1:300	17 rpm	Yes	Aggregated after 3 hours
PBCA	Acetate buffer pH 4.0	1:50	17 rpm	Yes	Aggregated after 3 hours
PBCA	Acetate buffer pH 4.0	1:300	17 rpm	Yes	Aggregated after 3 hours
PBCA	Deionized water	1:50	17 rpm	Yes	No aggregation seen
PBCA	Deionized water	1:300	17 rpm	Yes	No aggregation seen
PBCA	Phosphate buffer pH 7.4	1:50	10 rpm	Yes	Aggregated after 24 hours
PBCA	Acetate buffer pH 5.5	1:50	10 rpm	Yes	Aggregated after 24 hours
PBCA	Acetate buffer pH 5.5	1:300	10 rpm	Yes	Aggregated after 24 hours
PBCA	Acetate buffer pH 4.0	1:50	10 rpm	Yes	Aggregated after 24 hours
PBCA	Acetate buffer pH 4.0	1:300	10 rpm	Yes	Aggregated after 24 hours
PBCA	Phosphate buffer pH 7.4	1:50	5 rpm	Yes	Aggregated after unknown hours
PBCA	Acetate buffer pH 5.5	1:50	5 rpm	Yes	Aggregated after unknown hours
PBCA	Acetate buffer pH 5.5	1:300	5 rpm	Yes	Aggregated after unknown hours
PBCA	Acetate buffer pH 4.0	1:50	5 rpm	Yes	Aggregated after unknown hours
PBCA	Acetate buffer pH 4.0	1:300	5 rpm	Yes	Aggregated after unknown hours
PBCA	Phosphate buffer pH 7.4	1:50	0 rpm	Yes	No aggregation seen
PBCA	Acetate buffer pH 5.5	1:50	0 rpm	Yes	No aggregation seen
PBCA	Acetate buffer pH 5.5	1:300	0 rpm	Yes	No aggregation seen
PBCA	Acetate buffer pH 4.0	1:50	0 rpm	Yes	No aggregation seen
PBCA	Acetate buffer pH 4.0	1:300	0 rpm	Yes	No aggregation seen
PBCA	Citrate buffer pH 5.5[3]	1:50	5 rpm	Yes	Aggregated after 3 hours
PBCA	Citrate buffer pH 5.5	1:300	5 rpm	Yes	Aggregated after 3 hours
PBCA	Citrate buffer pH 4.0	1:50	5 rpm	Yes	Aggregated after 3 hours
PBCA	Citrate buffer pH 4.0	1:300	5 rpm	Yes	Aggregated after 3 hours
PBCA	Cell medium	1:250	5 rpm	Yes	Tiny aggregates after rotation. Lots of aggregates when shaken
PBCA	Cell medium	1:250	0 rpm	Yes	No aggregation seen
PBCA	Blood serum	1:250	0 rpm	Yes	No aggregation seen
PBCA	Esterase + buffer pH 7.4	1:250	0 rpm	Yes	Sedimentation after few hours
PBCA	Esterase + buffer pH 7.4	1:250	5 rpm	Yes	No sedimenation, but some aggregates seen

[1] All PBCA particles come from batch YM 66

[2] All samples were stored in an oven at 37°C

[3] From the table it looks like the acetat buffers and the citrate buffers were evenly unstable. In the reality the citrate buffers seemed to be even more unstable than the acetate buffers.

Chapter 5

Discussion

5.1 Possible mechanisms responsible for mean size increase

The size of the particles is reported to decrease with time due to surface erosion in almost all available litterature.[41, 61, 70] The results of this project are not that straight forward. The mean size of the particles in buffers (with and without esterase) increases, in cell medium it is almost unchanged, while mean size of particles in blood serum actually decrease. (Figure 4.9a) For the particles that increase, they increase a few nm between each measurement, and the size-increase is faster with a high pH. Since the particles are growing 'incrementally', it is a clear sign that this process is not due to aggregation. In the case of aggregation the size would have increased much more.

Except for aggregation there are two possible ways that the mean size can increase. Firstly, due to swelling. The chemical reaction processes of the particles and thermodynamics can be used to explain this theoretically. Since surface degradation is found to be the main degradation pathway [70] for PBCA nanoparticles, acrylic acid-groups are created and water flows into the outer layer of the particle because of a change in the osmotic equilibrium. Acrylic acid is in contrast to an alkyl group hydrophilic, and water will therefore better mix with this polymer. Additionally, as the polymer is transferred to poly acrylic acid it is also becoming a polyelectrolyte ($-COOH \rightarrow -COO^- + H^+$). The charged groups will repel each other and water will fill the space. Water in the outer part of the particle will bring nucleophiles deeper into the particle which drives the degradation further on. Although the particles might increase in size the degradation is probably still a surface degradation phenomenon, in which alkyl groups on the surface are being degraded first. If the ion concentration in the solution is high, ions will diminish the electrostatic repulsion by screening the Debye layer (see equation (2.3)), and the concentration difference will not be large between the particle interior and the medium, with the result that the potential for swelling will be limited.

The second reason for an increasing mean size can be a particle size distribution change. If the concentration was not decreasing, the only explanation would have been that the size of the particles had increased. However, since the concentration is decreasing, there is another possibility. As surface degradation is the accepted theory of degradation, it is logical that smaller particles degrade faster than larger ones due to a larger surface to volume- ratio, which is seen earlier [70]. This size dependent degradation will result in a shifted fraction between large and small particles, and this can actually push the entire particle distribution mean size towards a higher value. As a matter of fact, if only the mean size was known particles could have decreased in size without beeing observed. Müller et al. [41] have also observed a size increase and explained it by a shift in the particle size distribution.

In the Appendix (section 7.2) a small examle is made in Matlab where the size of each particle in a distribution goes down, but the mean size goes up. This example is very simple, but it shows that it is possible that the mean size of a distribution goes up even though the size for each particle is reduced. This is possible only because the number of particles goes down, and shows the importance of measuring both the size and the concentration.

5.2 Initial size difference

The synthesized size for the PBCA and the POCA particles are close to 145 nm and 155 nm respectively, whereas the initial size of PBCA and POCA varied in different mediums (see Table 4.1). The first measurement was always carried out within 5 minutes after making, and there is therefore unlikely to believe that swelling was the reason for this increased size, because swelling is a reaction that happens after degradation. Several tests of particles in buffer solutions were made to ensure that there were nothing wrong with the original PBCA and POCA stock-solutions. The most likely reason is therefore that proteins, amino acids and other components that exist in cell medium and blood serum adsorbed onto the particle surface immediately after mixing. This effect is a known phenomenon, and very precise tests have been carried out to analyse this protein corona. Walkey et al. [72] found for instance 147 different proteins adsorbed on their gold nanoparticles when placed in a physiological environment. Beside, it is likely that some of these proteins tag the particles for destruction, by opsonisation. This new identity of the particles when entering physiological environment is something that one has to take into account when further tests are carried out.

It is interesting to see that the initial size change was so big between different mediums. Especially in blood serum, which is the most relevant medium. The adsorbed layer is about 45 and 35 nm thick for PBCA and POCA respectively, which is really a lot. Since the adsorbed layer in cell medium (with 10% serum) was significnantly lower than in blood serum, it points in the direction that mainly proteins attached on the surface. Beside, the fact that the adsorption on the POCA particles was smaller than for the PBCA particles, is a detail that is worth noticing. An important part of beeing an effective drug carrier, is namely to go unnoticed through the body, and this protein adsorption is definitely playing a role in

this process. The reason why the adsorption was smaller for POCA can be either because of better PEG-shielding, or because of the alkyl-type (octyl vs butyl). POCA particles are more hydrophobic than the PBCA particles because of the octyl chain compared to the butyl chain. The immune response is found to be more active towards hydrophobic particles.[43] Based on this information the POCA particles should have a thicker layer of adsorbed proteins. However, since this was not the case, the most probable reason may be that the PEG-shielding was more effective for the POCA particles. Another study on similar particles revealed a higher PEG-density on POCA particles. [75] However, it was not exactly this POCA type, so it may not be relevant.

There might be that the adsorbed protein layer has a stabilizing effect against degradation because nucleophiles and enzymes have a rougher way to come in contact with the polymer chains. The results of the cell medium enhances this hypothesis, whereas the results of the blood serum point in the other direction.

5.3 Behaviour of particles in different mediums

5.3.1 Degradation in buffer pH 7.4

The mean size in all the buffers, and especially for PBCA particles at pH 7.4, was significantly increasing (Figure 4.10a). This seems to be a swelling phenomenon since the number of large particles (>180 nm) increased. This effect was also seen for cell medium after 3.5 hours (Figure 4.11a), although it was more distinct in the buffers. Additionally, from these figures one can see that the major part of the smaller particles (<150 nm) disappear after a while. These two observations together point in the direction that two things happen simultaneously with these particles. On one side, they become surface degraded which reduces the size. On the other side, there is swelling, which increases the size. Even though swelling is only observed for larger particles it does not mean that it does not happen for smaller particles. An interesting detail regarding swelling, is that it is only observed in the second and third measurements after degradation has started. For instance in Figure 4.3c most of the observed swelling was seen after 3.5 hours. The swelling contiuned at least until 9 hours, but this was observable just for a tiny part of the particles. Thereafter no swelling was observed and it seems like the particles have reached their swelling limit. The mean size continued to increase, but this was not due to observed swelling.

My hypothesis is that the largest particles, those with the lowest surface to volume ratio, degrade slowest, which agrees with earlier observations. [70] The water intrusion into these particles are allowed to happen, and they have a small netto increase in size. The smaller particles, in which the surface area to volume is high, the degradation will in turn happen faster. The particles will perhaps swell a little bit, but the shrinking due to surface erosion is more dominant, and the netto size is decreasing. Hence, the peak of the size distribution is getting smaller and shifted to the right, which results in a increased mean size and STD.

For POCA particles, one can see that the particles have sweelled a bit(Figure 4.13a), but there no large concentration drop was observed. The concentration was rather slightly increasing, before it stabilized. The reason for this is not known, but it may be due to the preparation method, see section 5.8.2. Anyway, it is clear that degradation is not a fast process for POCA particles. Swelling will happen only after degradation, and since the degradation here is lower, the swelling will in turn be less observable.

5.3.2 Degradation in blood serum

Already at the first measurement one could clearly see that the behaviour of nanoparticles was different in blood serum than in buffer pH 7.4. The initial size and the STD was much bigger both for PBCA and POCA (Figure 4.10b and Figure 4.13b). Since the first measurement was taken only minutes after sample-making, it is likely that the layer of adsorbed proteins was unevenly thick from particle to particle. This is possibly the reason for a very high STD and a high mean size. In blood serum the STD decreased pronouncedly over time, which mean that the particle distribution went from beeing slightly polydisperse to more monodisperse. One explanation might be that these proteins had time to adsorb more homogeneously, which then lead to a decrease in the mean size and a reduction in the STD.

The concentration decay for PBCA in blood serum and in buffer pH 7.4 was almost identical (Figure 4.9b). However, if one looks at the particle distribution curves (Figure 4.10), they are completely different. In buffer Ph 7.4 the distribution became very wide and flat, which resulted in a very large STD. In blood serum, on the other hand, the width of the particle distribution became smaller and smaller and the peak height was kept relatively high. This behaviour indicate that the particles in blood serum did not increase in size because of swelling. However, swelling was expected to be less dominant in blood serum due to the high ion concentration present (see section 2.4.2), and this may be the explanation why the particle distribution in those two mediums behaved so differently.

PBCA particles are earlier tested in blood serum [57], in which the particles were found to be unchanged after 6 days in blood serum in vitro. These results, however, do not coincide with our results. This can be explained simply because the particles are not the same. There are so many variables that can be different, like synthesis parameters, average molecule weight, PEG-density etc.

POCA particles (Figure 4.13b) turned out to be more robust particles and in buffer pH 7.4 not much was happening. In blood serum, on the other hand, there were clearly changes, but not so large as for the PBCA particles. Just as for PBCA, the size distribution width and the intial size was larger than in buffer pH 7.4. The distribution went also towards a more monodisperse distribution. In contrast to PBCA, the peak height became higher as the time went on. This must mean that more and more particles were getting close to the peak size, which only was possible if no or few of the particles in this size region degraded

and larger particles decreased in size until they reached this peak value. The most probable reason for this must be that the POCA particles over time lost their proteins adsorbed on the surface. However, an accurate protein analysis [72] is neccessary in order to know if this hypothesis is true. Beside, it must be pointed out that the final blood serum possibly did not hold the same quality as the blood serum initially. Many of the components become changed after a short time [8]. These changes happen only in vitro and can cause effects that not will happen in vivo.

5.3.3 Degradation in cell medium

For PBCA and POCA the intial mean size (Figure 4.9 and Figure 4.12) of particles and the STD in cell medium were larger than for particles in buffer, but smaller than in blood serum and esterase. During 5 days of degradation the size and the STD fluctuated and there was no clear pattern. Nevertheless, this was the medium in which the PBCA particles degraded least. Since many uptake studies in vitro use cell cultures and cell medium, it is a good thing that the cell medium itself did not affect the particles much.

It is surprising that the concentration did not decrease faster in cell medium than in a regular buffer system. Müller et al. [42] found a faster degradation in cell medium than in a phosphate buffer, and explained this by the presence of 5% fetal bovine serum (FBS), which contributed with an enzymatic degradation [59]. Since the cell medium used in this project contains 10% FBS, a faster degradation was thought to take place. Perhaps there are substances in cell medium that effectively stabilize the particles and prevent degradation. In the Appendix (section 7.4) there is an ingredient list of all the different components in the cell medium. To know which one of these that might be responsible for this behaviour is difficult. It is logic to think that these two cell mediums are approximately the same, and the outcome was therefore expected to be quite similar. However, as mentioned other parameters like PEG-density and protein adsorbance can effect the degradation as well.

After 3.5 hours the entire particle size distribution for PBCA was shifted towards a higher value. The concentration was unchanged while the mean size increased. This detail looks definitely like a swelling behaviour, even though it is not that clear as for particles in buffer pH 7.4. However, this was expected because of the presence of more ions, and this observation enhances the swelling theory.

5.3.4 Degradation in buffer pH 7.4 with esterase

In general a more rapid degradation is shown for PACA particles when esterase is present. [54] Esterase is a hydrolase enzyme that makes esters to split into an acid and an alcohol, and is found to catalyse the degradation process of PACA particles (see more in section 2.4.5). Based on this information a faster degradation was expected. However, the degradation in the buffer-esterase medium turned out to be very different from the other experiments. (Figure 4.11b and Figure 4.14b)

The STD of the PBCA particles in esterase was initially high. This behavior was probably the same as for particles in blood serum, except that esterase was the only protein adsorbed. The particles sedimented within few hours (Figure 4.8). By looking at equation (2.1) and Figure 2.10 one can see that the sedimentation velocity for particles in the 100-200 nm range is very slow (approx. 1mm/day). Since sedimentation was observed after a few hours (Figure 4.8), and such quick sedimentation happen only when particles are large ($> 1\mu m$), this means implicit that particles were aggregated.

Even though the samples were homogenized by a Vertex rotator before each measurements, it is likely that the Nanosight measurement were affected by this sedimentation. Aggregates could clearly be seen in the viewing field. Even though areas with many aggreagates were tried to be avoided during video capture, some of them were included in the measurements. The presence of aggregates have therefore possibly affected the degradation process and the measurements, since the concentration within the first 24 hours was measured to have increased to 160% for PBCA and 240% for POCA. (Figure 4.9b and Figure 4.12b) The high concentration is hard to explain, but perhaps the adsorption of esterase somehow made the particles more visible for the tracking system of the Nanosight, or that a concentration gradient occurs in the sample due to sedimentation, in which the concentration is more dense at the place where the $20\mu l$- nanosight sample was picked.

After 24 hours the samples were installed in a rotating device to avoid sedimentation. The measurement after this point behaved more as expected. The concentration decreased rapidly for both PBCA and POCA particles the next 24 hours. The particle distribution became very flat and low. However, after this point not much was happening. It is clear that the esterase present had an effect on the degradation, but since the experiment turned out to be so affected by this sedimentation it is not easy to tell exactly how the esterase worked.

5.4 pH dependence on degradation

5.4.1 pH dependence on degradation of PBCA particles

From Figure 4.2 it becomes clear that the pH affected the degradation of the PBCA particles. The higher pH the faster was the concentration decay, and the more the measured mean size of the particles increased. Except for the size increase, this is in accordance with earlier observations [57]. The fact that particles were so to say unchanged still after a month in a pH 4 buffer is a good thing, since the particles are stored at this pH. The degradation at pH 5.5 was not fast, but there was at least some. This is an important feature if the nanoparticles happen to end up in the lysosomes. For pH 7.4, which is the highest pH the particles will be exposed to in the body, the degradtion was significant. This is both good and bad. 37% of the particles in buffer pH 7.4 have been completely degraded within the 3.5 first hours. Since the particles must pass through this pH before they are able to enter the cell, there is a chanche that a great fraction of the nanoparticles are degraded before they reach the cell. On the other side, it is positive that all the particles do not degrade

within an hour, then none of the particles would have been left to enter the cancer cells. Additionally, clinically a large part of the nanoparticles will be assembled in a microbubble structure[1]. Even though no tests are carried out on this, it is likely that these nanoparticles will be more protected towards degradation than what free nanoparticles are.

In buffers this size increase is believed to occur due to swelling and a particle distribution shift. Swelling is observed mainly for PBCA particles in buffer pH 7.4 (1:500 and 1:250) in Figure 4.3. However, it is believed to happen also in pH 5.5 an pH 4.0, but the process happened much slower here, and it was therefore not observed.

The concentration decay for PBCA particles in buffer pH 5.5 and pH 7.4 seemed to follow an $\frac{1}{x}$ -decay. This statement is based on curve fitting in Matlab, see Figure 4.5, with a rapid decrease in the beginning followed by a more gradual decrease. The reason why this behaviour was seen may be due to the size dependent degradation. In the beginning there was a large fraction of particles with a high surface to volume ratio. Many of these small particles degraded very fast, and the number of particles that disappeared (degraded completely) per time unit was very high. As the time went on, this fraction between small particles and large particles decreased continuously, and there were fewer particles that could disappear per time unit.

For PBCA 1:300 and 1:500 in buffer pH 7.4 a sudden drop in the mean size could be seen. From Figure 4.3 one can see that the reason simply was a particle size distribution shift, in which the distribution went from beeing quite polydisperse to more monodisperse. The two last measurements for the PBCA pH 7.4 sample were carried out with a higher gain in order to actually see some particles. This increased gain has possibly increased the particle concentration higher than what it actually was, but the size distribution may actually be a more real picture of the system, since a larger number of particles are counted which results in a better statistical analysis. Otherwise, if this behaviour is not caused by parameter settings, it must mean that large particles suddenly degrade faster than smaller ones, which is rather unlikely. Instead of changing the gain, the samples should have been diluted less, and the value corrected afterwards.

Nevertheless, for both samples this size drop happended after approximately 10 days when approximately 10% of the particles were remaining. So no matter what happens, it is possibly not important for the behaviour of the particles when placed in the body. Anyway, these results supports the theory that surface degradation happen and particles decrease in size.

5.4.2 pH dependence on degradation of POCA particles

The POCA particles on the other hand, did not increase much in size in any of the pH-buffers, and the concentration followed the same pattern for all pHs. The trend is clear,

[1]The nanoparticles are supposed to stabilize micron-sized gasbubbles

namely that POCA particles in this project are not affected much by pH differences. Based on the available litterature, one should have expexted a similar pattern as seen for PBCA, just slower. The most probable reason for this behaviour is that the octyl-groups shield the ester group so effectively that nucleophiles are not able to reach the ester-carbon, with the result that degradation is slowed down and finally stops. A higher pH means more nucleophiles, but also the increase of nucleophiles seems not to be able to penetrate the shielding from the octyl-groups. Based on these results there may be possible to tune the life time of particles, which is very valuable in the drug delivery industry. This experiments gives a good picture of the situation, but one should be careful of drawing bastant conclusions based on one experiment. This behaviour could also be due to other parameters like the PEG-layer, the molecular weight [70] of the polymer chain or a parameter that at this time is not known.

5.5 Analysis of degradation by scanning electron microscope

A few images of the degradation process were taken by a scanning electron microscopy. The PBCA particles (Figure 4.15) are initially perfectly smooth and spherical, and no surface reaction seems to have taken place. Additionally, they are clearly flocculated. This is, however, a result of the drying process, and is not the real situation in a medium.

On the second image, taken after 5 days, one can clearly see that the particles are more melted together, indicating that something has happened on the surface, and made them more sticky.

On the third image, taken on day 11, no nanoparticles can be seen. This image has another scale bar, but this is done on purpose to show that no particles can be seen over the entire area. A probably reason why particles cannot be seen at day 11 is possibly that most of them are degraded, which coincide with the concentration curves from the Nanosight measurements. However, from the curves there should be some particles left. The reason why those cannot be seen may be due to swelling. The particles remaining must initally have been very large. It is likely that a large fraction of the particles now consist of a polyacrylic acid bound to water. When a water filled particle dries the water naturally evaporates, while the polymer skeleton remains. However, the skeleton does not anymore have the strength to keep a spherical shape and will just collapse. If one look at the picture in detail, one can see some mushy substances. This may be the collapsed polymer skeleton.

When looking at the POCA particles (Figue 4.16) they show a completely different behaviour. Within 11 days no significant change were seen. The particles look in fact bigger, but based on a few particles this is not enough to say that this is valid for all the particles. Beside, the scale bar between them is slightly different. Nevertheless, the main purpose of these images was to show that something happen to PBCA, whereas POCA is almost unhanged.

All in all, these images coincide quite good with what is seen from other instruments and strengthen the validity of the other results.

5.6 Detection of degradation by Gas Chromatography

Degradation products from PBCA 1:25 and 1:250 and POCA 1:250 were detected and quantified by GC (Figure 4.17. In this experiment one assumes that ester-hydrolysis is the only degradation pathway (see Figure 2.9) and by quantification of the degradation products butanol (for PBCA) and octanol (for POCA) one can predict the degradation-progress.

Octanol was detected for particles degraded in NaOH, but not detected for degradation in buffer pH 7.4. This points in the direction that no octanol was present in the buffer solutions, or at least so litte that it was not measureable, which again means that no particularly degradation has taken place. This result is not surprising at all based on the results from the Nanosight.

Huang and Lee [27] have done tests on nanoparticles made of PBCA, POCA and a copolymer of PBCA/POCA. Their results coincide quite good with our results. The butanol/octanol yield followed a linear pattern, just as our curves. Huang and Lee used a concentration of 2mg/ml which is approximately the same as our 1:25 particles (2.24mg/ml). After 2 days a butanol yield of around 1% is found. This is remarkably similar as what is seen for our 1:25 particle solution. Our 1:250 solution lies well above this level with a linear release of butanol reaching 26% yield after 2 days. For POCA particles they measured an octanol yield first after 10 days. Since our experiment did not last that many days one cannot say if this coincide perfectly with our results, but the fact that no octanol was detected within 2 days is at least comparable.

Sullivan and Birkinshaw [61] carried out GC experiments with the presence of esterase. Their nanoparticle concentration was relatively high as well, namely 2mg/ml. They detected a butanol that was much greater than our results. They observed a biphasic release, in which 12% butanol yield was reached after just 1 hour. Then a more gradual linear increase was seen the next hours with approximately 20% butanol yield after 4 hours. The biphasic phase is believed to occur because esterase at the beginning is not diffusionally hindered to reach the esterase groups. Degradation of ester-groups deeper into the particles is, on the other side, diffusionally hindered because the esterase molecules are quite big. This butanol release is significantly higher than our results, but one has to keep in mind that our experiments were carried out in a buffer only, so a slower degradation is expected.

A detail that is noteworthy, is the significant difference between the 1:25 particles and the 1:250 particles. Last year, experiments [4] pointed in the direction that the concentration may have an influence on the degradation-rate, and here is a another experiment that points in that direction. It would have been interesting to see an even more diluted sample, but

the sensitivity of the GC may not be good enough.

The degradation of the two PBCA samples lie well below the degradation measured by Nanosight. After 40 hours approximately 25% degradation is measured in GC. From the Nanosight results the particle concentration decreased rapidly the first 10-15 hours, and after 40 hours only 30% of the particles were remaining. Based on these numbers it is intiutive to think that one of the methods are showing the wrong result. However, one has to keep in mind that these two methods are not directly comparable. On one side, the Nanosight measures the concentration decay of particles. However, a 10% decrease in the particle concentration does not implicit mean that 10% of the mass is degraded, because small particles are believed to degrade first. The GC on the other hand, measures something completely different. It measures the butanol concentration of the sample, which tells us how many percent of the ester-groups that are hydrolysed. Each monomer that is hydrolysed contribute with one butanol molecule, and thereby one can calculate the degree of degradation. A 100% butanol yield corresponds to a 100% degradation of the ester-groups. The amount of butanol is related to the total polymer-mass that is degraded and not the number of particles. It might be possible to transfer the particle concentration decay from Nanosight to a volume or mass decay. But since the particles seem to swell, this may disturb the result. Despite the differences between these methods, the butanol yield was expected to be larger based on the results from the Nanosight.

The drawback by the GC-results is the fact that only one paralell for each concentration is completed. This makes the results insecure, even tough they coinicide with the results of Huang and Lee. To find out whether or not these results are valid, more samples over a longer time period have to be measured. Additionally, the way the butanol yield is calculated is a bit insecure, because the maximum amount was only detected once. A calibration curve had been better, in which several samples with a known butanol-mass had been plotted. The problem with a calibration curve is to get it accurate due to the very small amounts of butanol needed. (0.01 - 0.54mg for 5ml diethylether)

The maximum amount measured by GC is not equal to the maximum theoretical amount calculated. The maximum theoretical value is calculated to be $7.3 \cdot 10^{-6}\, mole$ for butanol and $4.0 \cdot 10^{-6}\, mole$ octanol for 5ml of 1:250 solution (see Section 2.7.3). The amount of butanol and octanol measured is calculated to be $2.7 \cdot 10^{-6}\, mole$ and $2.5 \cdot 10^{-6}\, mole$, respectively.

It is a good thing that they lie below the theoretical values, but ideally they should have been closer to the theoretical values. There could be many reason why these two values do not coincide. Firstly, the theoretical value assume that all ester-groups are present in the stock solution, and that degradation happen through ester-hydrolysis only. This might not be the case. The degradation could have happened already in the synthesis as far as we know. Secondly, as mentioned in the theory (Section 2.4) there are several possible degradation pathways. Ester-hydrolysis is found to be the main one, but the other could be present to some degree, which makes the theoretical outcome wrong. The third reason may be that all the butanol has not been extracted into the diethylether. The fourth reason is the

fact that the calculation of the stock solution density is based on a dryweight measurement. In addition to PBCA this dryweight contains fractions of PEG, surfactants etc, which will shift the teoretical mass of butanol to be slightly higher than what it actually is. The fifth reason is that the mass of butanol is calculated through the known pentanol mass. This is believed to be a relatively good way, but not the best way. The best way would have been to compare with a calibration curve of known butanol and octanol values.

5.7 Importance of particle concentration for degradation and measurements

In the litterature there is not a general way how a degradation test should be carried out, and especially the concentration varies a lot. In GC experiments for instance, the particle concentration is unrealistically high (2mg/ml [27, 61]) compared to the concentration that will find place in the body. Cellular uptake studies is limited to approximately $20\mu g\,/ml$ due to toxicity concerns [37]. Between these two concentrations regimes there is a 100 fold difference. The particle concentration is likely to have an affect on degradation. This assumption is made on the foundation of several reasons. Firstly, it is likely that a dense concentration faster will reach some kind of an equilibrium with respect to degradation products. Octanol for instance is not soluble in water. Octanol dissolved in water will be a very unfavourable energetically system, and an equilibrium is likely to be established in a concentrated solution. However, in a very diluted sample the amount of solvent is much higher and the system can almost be regarded as a dynamic system in which degradation products are removed from the vicinity of the particles. Secondly, the surface energy of the system becomes larger the more particles that are present, and the driving force for aggregation becomes mutually large (Figure 2.11). It is shown [4] that degradation for aggregated particles happens only at the surface, whereas bulk particles remain unchanged. The GC-measurements (Figure 2.21) between 1:25 and 1:250 show a significant difference in the degradation rate, but due to only one paralell for each of the samples, this is a result that must be taken with a dose of suspiciousness. Nevertheless, this is a relation that is important to find out, since the concentrations between degradation measurements vary very much.

In the Nanosight as well, the concentration is crucial. Perhaps for degradation, but especially for measurements. Since the degradation measured by Nanosight relies on the concentration of particles it is really important that the obtained concentration actually is the right one. Otherwise this measurements are meaningless. Fortunate, the concentration has been proven [18] to be quite accurate when diluted right.

Zetapotential measurements are also vulnerable for the particle concentrations. The zetapotential changes namely with the particle concentration to a certain point. After this point further dilution has no effect. This was seen in own observations (Table 4.2), but is also confirmed by others.[40] The reason for this is suggested to be because of the large surface

area of the nanoparticles which need a high number of ions per volume. In our system with 0.01 M phosphatebuffer, the number of ions is possibly not high enough to screen the charge of all particles, and the zetapotential becomes higher than what it is supposed to be. As the particle concentration goes down, a higher number of ions become available per nanoparticle, and the charge is then more screened which results in a zetapotential closer to zero.

5.8 Evaluation of methods and instruments for detection of degradation

5.8.1 Nanosight vs Zetasizer

To use Nanosight to detect degradation is something quite new. In the litterature the Zetasizer is the main instrument used for size measurement over time. However, especially for degradation tests the Zetasizer has many drawbacks compared to the Nanosight. This can be confirmed by own results (Figure 4.18), but also by others. [18] The conclusion is that Nanosight gives a more accurate particle size and especially in polydisperse samples. It is less influenced by the presence of large particles and give a good particle concentration when diluted right. The drawbacks for the Nanosight is the need for a skilled operator which can manually optimize the parameters in order to get the best results. The Zetasizer is more like a push-and-play device in which the operator is less able to have an impact on the result quality.

However, the main advantage of the Nanosight over Zetasizer is the fact that one can see the size changes together with the concentration. In this project it would not have been easy to tell if particles were degraded or not based on the mean size only.

5.8.2 Dialysis-cassette method vs reagent bottle method

All in all, the behaviour of particles in the two different setups were comparable, even though the results not were perfectly overlapping. The degradation was faster in the reagent bottle method, but the temperature was also higher, which is shown to speed up the degradation. [59] The main advantage of the dialysis cassette is the fact that it mimics physiological processes better than the reagent bottle. The exchange of surrounding fluid removes the degradation products, which is exactly what is happening in a body. The two main drawbacks are the lack of rotation that can lead to sedimentation (for long time testing) and the large amount of medium that is needed. Additionally, it is hard to find an oven that is big enough for many samples. This makes it unappropriate for testing at 37°C. Mediums like blood serum, cell medium and esterase are quite expensive, and it is not appropriate to use a litre of medium for each sample per week. The reagentt bottles are exactly the opposite. They lack the ability to exchange the fluid, but they are very mobile and can easily be placed in an oven or rotated. However, there is possible to utilize the best properties of both in

one single setup. There are dialysis cassettes that could be mounted inside a regular reagent bottle. They would probably have been the ultimate choiche for this experiment.

Another thing that distinguish these methods is the way a sample is prepared before the Nanosight-measurement. For both a small amount is taken out and diluted with deionized water. In the dialysis cassette the total sample volume is so large (28 ml) that it does not matter for the remaining sample if 0.1 ml or 1ml is taken out for each measurement. If a tiny inaccuracy is present in this withdrawal, it will not affect the concentration much. On the other hand, in the reagent bottle method which only contains 5ml, it matters a lot how much is taken out. If 1 ml of sample is taken out for each measurement, there would after a few measurements not be more left, and the sample is destroyed. In order to leave the original sample unchanged, an amount of only 20µl was withdrawn. However, when such small volumes are taken out, just a tiny inaccuracy can make a large concentration difference, and since several POCA-measurements from this method had a concentration slightly higher than 100% (the esterase-samples do not count here), the reason can simply be inaccuracies in the withdrawal process. Even though a micro pipette is used, some sample residues will always be left in the pipette tip, and a small difference can possibly be enough to shift the concentration slightly. For future work it is therefore recommended to take out more than 20µl for each measurement. A larger sample than 5 ml is then in turn also needed.

5.9 Final remarks

The drug carrier route inside the body goes first through the blood vessels. The concentration of PBCA will quickly degrade in this environment. Forunately they will not stay here for ever[2]. Eventually they will enter the tumor tissue. The environment in the tumour tissue is likely less aggressive, and the pH is probably somewhat lower than in the blood vessels. However, the degradation will probably go on, just slower. If the particles finally become internalized in the cancer cells, they are believed to end up in lysosomes [25], in which the pH is around 5.[5] A low pH will decrease the degradation rate. On the other hand, enzymes like esterase in the lysosomes is believed to accelerate the degradation process. Which one of these contributions that are most dominant are not known. Nevertheless, if the nanoparticles actually happen to end up in the lysosomes, it seems like the degradation of PACA particles are greater outside of the cells.

Drug release tests are not carried out in this project, but if it turns out that the degradaton happen simultaneously with the degradation, these POCA particles are not appropriate because they degrade too slow. PBCA has a relatively fast degradation rate, but a fast degradation rate seems to correlated with toxicity,[27, 37] and Westrøm [75] found that for our particles, PBCA particles are distinctly more toxic than POCA particles. These two in-

[2]The exact time for our particles are not known, but for instance for DOXIL, only 10% of the particles were left after 7 days. [20]

formations tell us that neither the PBCA nor the POCA particle happen to be the ultimate drug carrier. A new particle which utilizes the best properties from both PBCA and POCA must be synthesized. However, this is not an easy job, but it is naturally to start with either a copolymer of PBCA and POCA, or perhaps use a particle with an alkyl-length between butyl and octyl, for instance hexyl.

Chapter 6

Conclusion & Future Work

In this project we have seen that the Zetasizer instrument was not appropriate for detecting degradation of PBCA and POCA nanoparticles, because of its limitations for polydisperse samples. The Nanosight, however, gave reliable results for both monodisperse and polydisperese samples. In addition to calculate the mean size of the particle distribution the concentration of it was also generated, which turned out to be very valuable. The degradation products butanol and octanol were detected and quantified by the GC-instruments. This result gave supplementary information to the Nanosight results, and these instruments together give sufficiently information to understand many of the processes happening during degradation.

Through measurements it was shown that degradation of PBCA particles was correlated with the pH, in which pH 4 resulted in little or no degradation, pH 5.5 some degradation, whereas in pH 7.4 a pronounced degradation was observed already after 1 day. For POCA, the degradation was similar in all pHs. The particles were further tested in cell medium, blood serum and with the presence of esterase. PBCA degraded relatively fast in all of them, in which cell medium was the slowest one. POCA particles were not much affected by none of these mediums, but some degradation was seen in blood serum and in buffer pH 7.4 with esterase.

In buffers a significantly mean size increase was seen, and based on the raw datas available it is very likely that this behaviour was caused by swelling. Swelling was observed mainly in buffer pH 7.4, but also to some degree in cell medium, but not in blood serum. The reason why swelling was not equally present in all mediums was possibly because of the high ion concentration in cell medium and blood serum, which diminished the driving force for swelling. In blood serum the particle mean size was found to decrease. This result coincided better with earlier studies.

Surface erosion is still thought to be the main degradation pathway, and small particles are believed to degrade faster than larger ones due to the difference in surface to volume ratio. This size dependent degradation and swelling seemed to be responsible for the increased

standard deviation that was observed in buffer systems. In blood serum the STD started initially high and decreased over time. The initially high STD came from proteins that adsorbed immediately at the surface. The adsorbed layer was largest in blood serum, which also had the highest amount of proteins. The adsorbed layer seemed to erode over time, especially for the POCA particles. This conclusion is based on the fact that the STD decreased while the concentration was maintained high. However, one has to keep in mind that things may have happened with the blood serum which have affected the behaviour in vitro that not neccesarily would have happened in vivo.

An experiment with esterase was also carried out, but due to sedimentation, no conclusive results were obtained.

All in all, the main observations of this experiments were in compliance with available litterature. The degradation increaseed with increasing pH as described in the litterature.[35, 59, 70] Particles with a longer alkyl chain is reported to degrade slower than similar particles with a shorter alkyl chain.[27, 41] This is exactly what was seen in this experiment. The one thing that was surprising, was the fact that POCA particles were not observed to be affected by the pH. Based on the available litterature, one should have expected a similar pattern as seen for PBCA, but a more slowly development.

Results that point in the direction that particles swell and increase their size for PBCA particles are rarely reported. The results from this project did not only show swelling, they showed that particles decreased their size simultaneously with swelling which never is reported earlier.

6.1 Future Work

For future work the relation between the concentration and degradation must be inspected. Additionally, some of the experiments in this project were carried out with just one paralell or did not go as planned. To do some of this over again will increase the reliability of the results, and possibly give us answer of some of the questions and uncertainties that did arise during this project.

In order to make a drug carrier with a long circulation time that is not so easy recognized by the MPS, a better PEG-shielding is required. Analysis and more testing on this protein corona that attached on the particles when placed in blood serum could be helpful in order to solve this circulation problem.

Finally, tests on the actual drug release must also be done sooner or later, in order to see if the drug release correlates with the degradation.

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Chapter 7

Appendix

7.1 Debye length in blood

$$\lambda_D = \frac{1}{\kappa} \qquad \kappa = \sqrt{\frac{e^2}{\epsilon\epsilon_0 k_B T} \sum_i c_i^0 Z_i^2} \tag{7.1}$$

In human blood plasma, i.e blood without red and white blood cells and thrombocyts, contains 143 mM Na^+, 5 mM K^+, 2.5 mM Ca^{2+}, 1 mM Mg^{2+}, 103mM Cl^-, 27 mM HCO_3^-, 1mM HPO_4^{2-} and 0.5 mM SO_4^{2-}. The Debye length can be calculated based on this information.

$c^0_{Na} = 861 \cdot 10^{23} m^{-3}$	$Z_{Na} = 1$	$c^0_K = 30 \cdot 10^{23} m^{-3}$	$Z_K = 1$
$c^0_{Ca} = 15 \cdot 10^{23} m^{-3}$	$Z_{Ca} = 2$	$c^0_{Mg} = 6 \cdot 10^{23} m^{-3}$	$Z_{Mg} = 2$
$c^0_{Cl} = 620 \cdot 10^{23} m^{-3}$	$Z_{Cl} = -1$	$c^0_{HCO_3} = 163 \cdot 10^{23} m^{-3}$	$Z_{HCO_3} = -1$
$c^0_{HPO_4} = 6 \cdot 10^{23} m^{-3}$	$Z_{HPO_4} = -2$	$c^0_{SO_4} = 3 \cdot 10^{23} m^{-3}$	$Z_{SO_4} = -2$

When these values are inserted into equation (7.1) we get a Debye length of 0.78 nm. This example is reproduced from Butt, Graf, and Kappl [9] page 46.

7.2 Matlab-modelling

I have made a small particle example which shows that it is possible that the mean size of a distribution goes up even though the particle size of all particles is reduced. I start with a particle distribution with sizes from 10,11,12.... up till ...198,199,200. The algorithm is programmed so that the particle size decrease faster for small particles than for the large one, because of the larger surface to volume ratio. Particles below 95 nm is reduced to the size of its own percent. For instance a 90 nm particle is reduced to 90% of its size, namely $(90 \cdot 90\% =)$ 81 nm, whereas a 50 nm particle is reduced to 50% of each size, namely 25. Particles between 95 nm and 150 nm decreases 2nm per iteration, whereas particles larger than 150 nm decreases 1nm. To reduce the particle number all particles that are below 10

nm are not counted. The mean size of the initial distribution is 105.0, the first iteration gives 107.6 and the second iteration is 115.9

This is a very simple example. The size distribution is not equal to the real particle distribution and the way the particles shrink is neither based on how the real particles shrink, but smaller particles shrink more than larger one. Although the examle is very simplified, it still fulfills its purpose, which is to proove that a mean size increase not necessarily is equal to a particle increase.

7.2.1 The functions

The functions below is written in MATLAB syntax, the first one (reduce) is used to reduce the size of the particles, the second (sizeaverage) is used to reduce the number of particles and calculate the mean size.

```
function reduce = reduce(distribution)

    for i=1:length(distribution)
        if distribution(i)<95
            distribution(i)=distribution(i)*distribution(i)/100;
        elseif distribution(i)<150
            distribution(i)=distribution(i)-2;
        else
            distribution(i) = distribution(i)-1;
        end

    end
   reduce=distribution;

return
```

```
function sizeaverage = sizeaverage(distribution)

    sizetotal=0;
    number=0;
        for i=1:length(distribution)
            if distribution(i)>= 10
                sizetotal = sizetotal + distribution(i);
                number = number +1;
            end

        end
    sizeaverage= sizetotal/number;

return
```

The size distributions and the mean size is then obtained by using the functions:

a = 10:1:200; % generates the particle distribution.

b= reduce(a); % reduces the size of the particle distribution.

c= reduce(b); % reduces the size of the particle distribution a second time.

mean-a = sizeaverage(a); % calculates the mean size of the distribution.

mean-b = sizeaverage(b); % calculates the mean size of the distribution and reduces the particle number.

mean-c = sizeaverage(c); % calculates the mean size of the distribution and reduces the particle number.

7.3 Calculation of the maximum mass of butanol and octanol from GC

The maximum mass of butanol and octanol for 5 ml of a 1:250 PBCA and 1:250 POCA nanoparticle solution. For the POCA solution, the pentanol area = 6240, and the octanol area = 816.
Added pentanol was 3µl, the density of pentaol is 811g/l. The total mass of pentanol added becomes then 2.5mg. The mass of octanol becomes then $2.5mg \cdot \frac{816}{6240} = 0.32mg \rightarrow \frac{0.32mg}{130.23mg/mole} = 2.5 \cdot 10^{-6}\, mole$

For the PBCA solution, the pentanol area = 572 and the butanol area = 48. The mass of butanol becomes then $2.5mg \cdot \frac{48}{572} = 0.20mg \rightarrow \frac{0.20mg}{74.12mg/mole} = 2.7 \cdot 10^{-6}\, mole$

The reason why the pentanol area differs between the samples is because of the split of the GC was changed. The split decides how much of the sample that goes through the column.

7.4 Cell medium

In Table 7.1 the ingredient list of the cell medium is listed.

Table 7.1: Overview of components in the cell medium

Components	**Molecular Weight**	**Concentration (mg/L)**	**mM**
Amino Acids			
Glycine	75	30	0.4
L-Arginine hydrochloride	211	84	0.398
L-Cystine 2HCl	313	63	0.201
L-Glutamine	146	580	3.97
L-Histidine hydrochloride-H2O	210	42	0.2
L-Isoleucine	131	105	0.802
L-Leucine	131	105	0.802
L-Lysine hydrochloride	183	146	0.798
L-Methionine	149	30	0.201
L-Phenylalanine	165	66	0.4
L-Serine	105	42	0.4
L-Threonine	119	95	0.798
L-Tryptophan	204	16	0.0784
L-Tyrosine	181	72	0.398
L-Valine	117	94	0.803
Vitamins			
Choline chloride	140	4	0.0286
D-Calcium pantothenate	477	4	0.00839
Folic Acid	441	4	0.00907
Niacinamide	122	4	0.0328
Pyridoxine hydrochloride	204	4	0.0196
Riboflavin	376	0.4	0.00106
Thiamine hydrochloride	337	4	0.0119
i-Inositol	180	7.2	0.04
Inorganic Salts			
Calcium Chloride (CaCl2-2H2O)	147	264	1.8
Ferric Nitrate (Fe(NO3)3"9H2O)	404	0.1	0.000248
Magnesium Sulfate (MgSO4-7H2O)	246	200	0.813
Potassium Chloride (KCl)	75	400	5.33
Sodium Bicarbonate (NaHCO3)	84	3700	44.05
Sodium Chloride (NaCl)	58	6400	110.34
Sodium Phosphate monobasic (NaH2PO4-2H2O)	154	141	0.916
Other Components			
D-Glucose (Dextrose)	180	4500	25
Phenol Red	376.4	15	0.0399

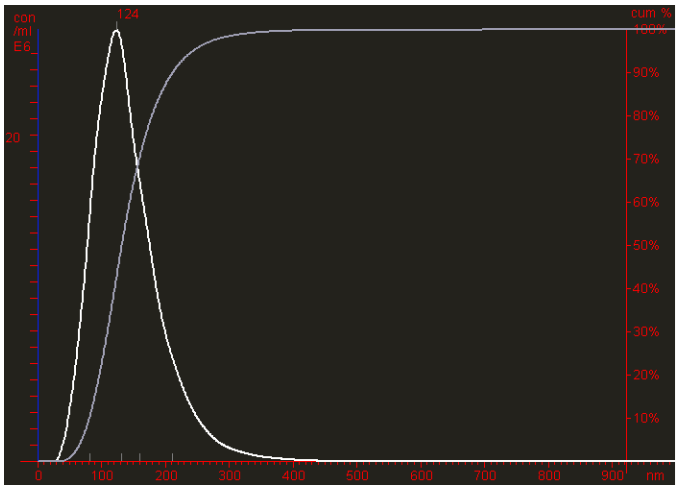
[1] The cell medium is also added 10% fetal bovine serum.

NANOSIGHT

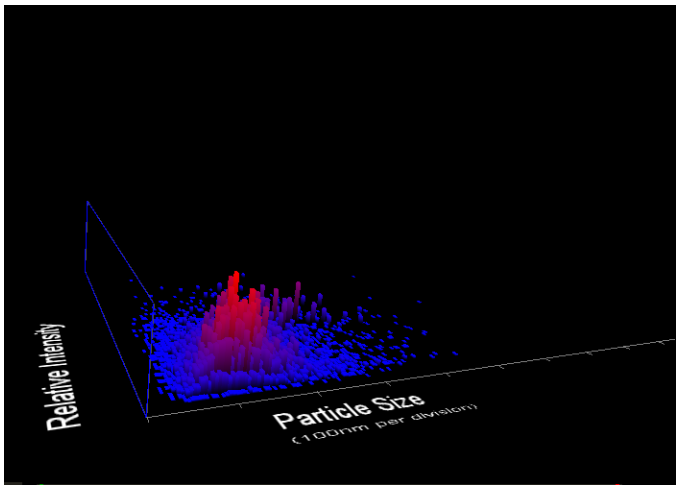
Nanoparticle Tracking Analysis (NTA) Version 2.3 Build 0025

ANALYSIS REPORT

Sample: PBCA
Date/Time of Capture:
Video File: sample 102 day1.avi analysis no: 001
Operator: Andreas
Comments:



Particle Size / Concentration



Particle Size / Relative Intensity 3D plot

Bin Centre (nm)	Concentration (E6 particles/ml)	Percentile Undersize (%)
10	0.000	0.000
30	4.927	0.170
50	61.592	2.289
70	192.177	8.902
90	367.311	21.542
110	487.608	38.322
130	512.538	55.960
150	402.812	69.822
170	297.233	80.050
190	200.045	86.934
210	134.320	91.556
230	89.024	94.620
250	56.160	96.552
270	34.838	97.751
290	21.453	98.490
310	14.244	98.980
330	9.583	99.310
350	6.428	99.531
370	4.465	99.684
390	3.098	99.791
410	2.109	99.864
430	1.421	99.913
450	0.958	99.945
470	0.625	99.967
490	0.373	99.980
510	0.212	99.987
530	0.135	99.992
550	0.099	99.995
570	0.070	99.998
590	0.042	99.999
610	0.019	100.000
630	0.007	100.000
650	0.002	100.000
670	0.000	100.000
690	0.000	100.000

Bin Centre (nm)	Concentration (E6 particles/ml)	Percentile Undersize (%)
710	0.000	100.000
730	0.000	100.000
750	0.000	100.000
770	0.000	100.000
790	0.000	100.000
810	0.000	100.000
830	0.000	100.000
850	0.000	100.000
870	0.000	100.000
890	0.000	100.000
910	0.000	100.000
930	0.000	100.000
950	0.000	100.000
970	0.000	100.000
990	0.000	100.000
1000-2000	0.000	100.000

Results

Mean: **142 nm**
Mode: **124 nm**
SD: **54 nm**
D10: **82 nm**
D50: **132 nm**
D90: **212 nm**
User Lines: **0 nm, 0 nm**
Concentration: **29.06 E8 particles/ml**
Completed Tracks: **13118**

Measurement Conditions

Temperature: 23.40 ^{o}C
Viscosity: 0.92 cP
Frames Per Second: 30.00
Measurement Time: 60 of 60 s
Drift Velocity: 325 nm/s
Camera Shutter: 30 ms

Analysis Conditions

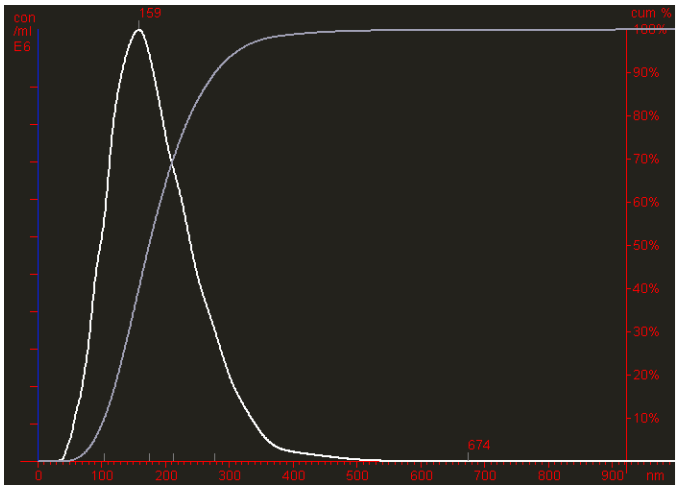
Blur: 3x3
Detection Threshold: 4 Multi
Min Track Length: Auto
Min Expected Size: Auto

NANOSIGHT

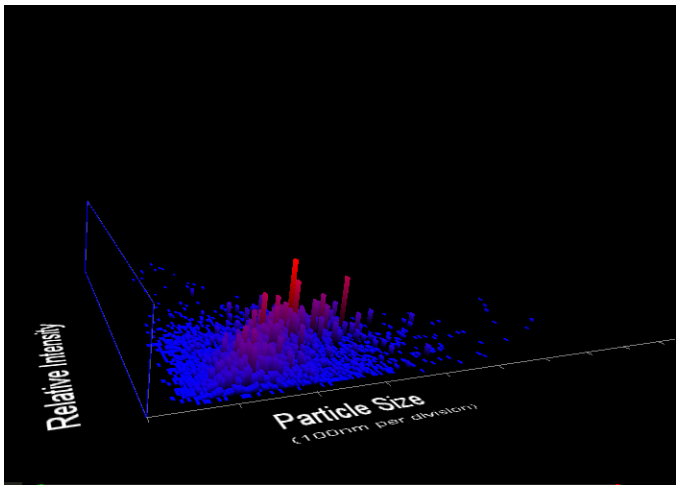
Nanoparticle Tracking Analysis (NTA) Version 2.3 Build 0025

ANALYSIS REPORT

Sample: PBCA 1:500
Date/Time of Capture:
Video File: sample 102 day2.avi analysis no: 001
Operator: Andreas
Comments:



Particle Size / Concentration



Particle Size / Relative Intensity 3D plot

Bin Centre (nm)	Concentration (E6 particles/ml)	Percentile Undersize (%)
10	0.000	0.000
30	0.377	0.021
50	11.073	0.638
70	40.521	2.895
90	92.410	8.041
110	148.107	16.290
130	202.854	27.588
150	226.804	40.220
170	224.131	52.703
190	194.806	63.553
210	162.391	72.598
230	134.429	80.085
250	102.618	85.800
270	79.428	90.224
290	57.958	93.452
310	39.431	95.648
330	26.645	97.132
350	16.074	98.027
370	9.131	98.536
390	6.075	98.874
410	4.776	99.140
430	3.836	99.354
450	2.971	99.519
470	2.163	99.640
490	1.460	99.721
510	0.947	99.774
530	0.615	99.808
550	0.394	99.830
570	0.260	99.844
590	0.212	99.856
610	0.237	99.869
630	0.299	99.886
650	0.362	99.906
670	0.394	99.928
690	0.381	99.949

Bin Centre (nm)	Concentration (E6 particles/ml)	Percentile Undersize (%)
710	0.325	99.968
730	0.245	99.981
750	0.163	99.990
770	0.095	99.996
790	0.048	99.998
810	0.021	99.999
830	0.008	100.000
850	0.003	100.000
870	0.001	100.000
890	0.000	100.000
910	0.000	100.000
930	0.000	100.000
950	0.000	100.000
970	0.000	100.000
990	0.000	100.000
1000-2000	0.000	100.000

Results

Mean: **186 nm**
Mode: **159 nm**
SD: **71 nm**
D10: **105 nm**
D50: **175 nm**
D90: **278 nm**
User Lines: **0 nm, 0 nm**
Concentration: **17.96 E8 particles/ml**
Completed Tracks: **6664**

Measurement Conditions

Temperature: 22.90 ^{o}C
Viscosity: 0.93 cP
Frames Per Second: 30.00
Measurement Time: 60 of 60 s
Drift Velocity: 819 nm/s
Camera Shutter: 30 ms

Analysis Conditions

Blur: 3x3
Detection Threshold: 4 Multi
Min Track Length: Auto
Min Expected Size: Auto

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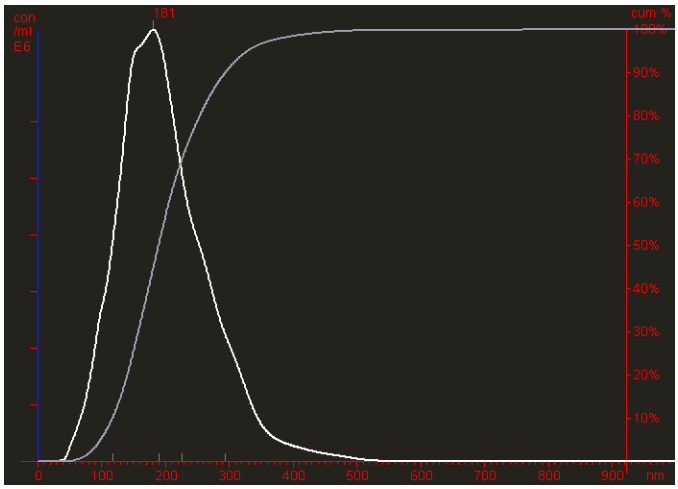
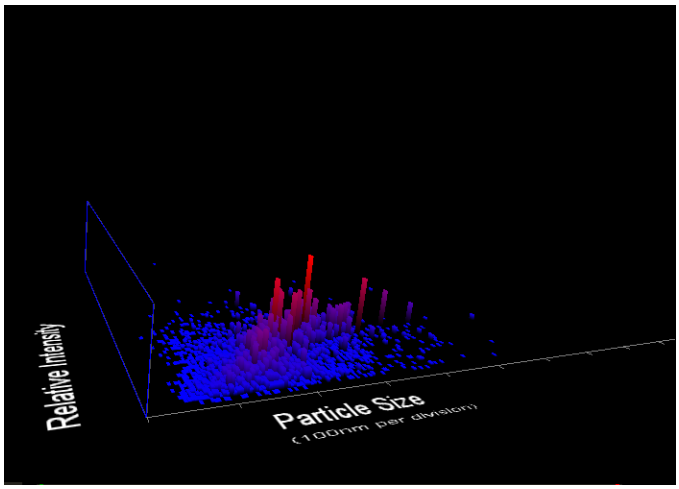
Nanoparticle Tracking Analysis (NTA) Version 2.3 Build 0025

ANALYSIS REPORT

Sample: PBCA 1:500
Date/Time of Capture:
Video File: sample 102 day4.avi analysis no: 001
Operator: Andreas
Comments:



Particle Size / Concentration



Particle Size / Relative Intensity 3D plot

Bin Centre (nm)	Concentration (E6 particles/ml)	Percentile Undersize (%)
10	0.000	0.000
30	0.201	0.017
50	4.632	0.406
70	17.179	1.848
90	39.511	5.164
110	65.137	10.632
130	101.089	19.117
150	139.821	30.853
170	149.703	43.419
190	148.971	55.924
210	126.826	66.570
230	98.655	74.851
250	79.659	81.537
270	64.274	86.932
290	48.373	90.993
310	36.427	94.050
330	24.079	96.071
350	13.969	97.244
370	8.774	97.980
390	6.424	98.520
410	5.011	98.940
430	3.830	99.262
450	2.912	99.506
470	2.171	99.688
490	1.421	99.808
510	0.775	99.873
530	0.393	99.906
550	0.244	99.926
570	0.212	99.944
590	0.207	99.961
610	0.185	99.977
630	0.137	99.988
650	0.081	99.995
670	0.038	99.998
690	0.014	100.000

Bin Centre (nm)	Concentration (E6 particles/ml)	Percentile Undersize (%)
710	0.004	100.000
730	0.001	100.000
750	0.000	100.000
770	0.000	100.000
790	0.000	100.000
810	0.000	100.000
830	0.000	100.000
850	0.000	100.000
870	0.000	100.000
890	0.000	100.000
910	0.000	100.000
930	0.000	100.000
950	0.000	100.000
970	0.000	100.000
990	0.000	100.000
1000-2000	0.000	100.000

Results

Mean:	**200 nm**
Mode:	**181 nm**
SD:	**71 nm**
D10:	**118 nm**
D50:	**190 nm**
D90:	**294 nm**
User Lines:	**0 nm, 0 nm**
Concentration:	**11.91 E8 particles/ml**
Completed Tracks:	**4024**

Measurement Conditions

Temperature:	22.80 ^{o}C
Viscosity:	0.94 cP
Frames Per Second:	30.00
Measurement Time:	60 of 60 s
Drift Velocity:	760 nm/s
Camera Shutter:	30 ms

Analysis Conditions

Blur:	3x3
Detection Threshold:	4 Multi
Min Track Length:	Auto
Min Expected Size:	Auto

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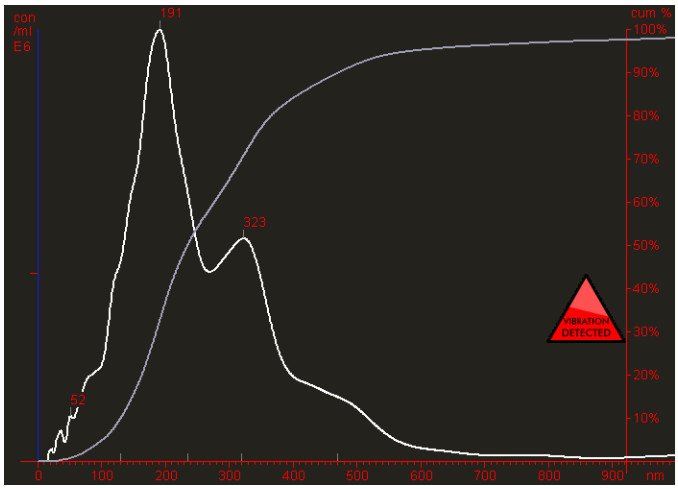
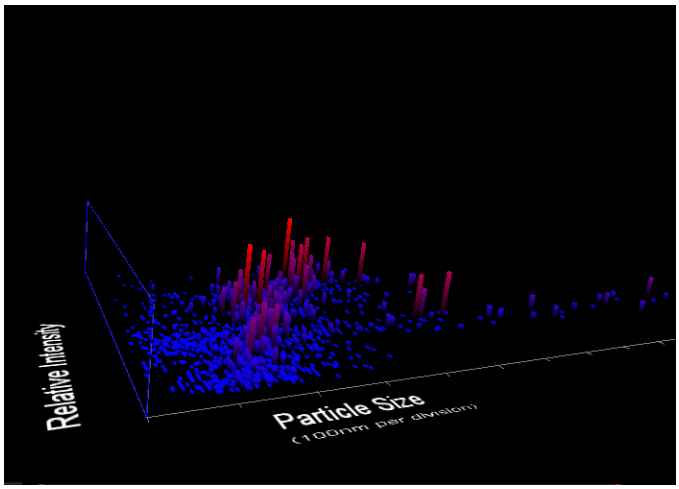
Nanoparticle Tracking Analysis (NTA) Version 2.3 Build 0025

ANALYSIS REPORT

Sample: PBCA 1:500
Date/Time of Capture:
Video File: sample 102 day10.avi analysis no: 001
Operator: Andreas
Comments:



Particle Size / Concentration



Particle Size / Relative Intensity 3D plot

Bin Centre (nm)	Concentration (E6 particles/ml)	Percentile Undersize (%)
10	0.123	0.026
30	2.123	0.473
50	3.776	1.269
70	7.136	2.772
90	9.586	4.791
110	14.039	7.748
130	21.648	12.308
150	29.279	18.475
170	39.222	26.737
190	45.585	36.339
210	39.713	44.704
230	30.973	51.229
250	23.723	56.226
270	20.478	60.539
290	21.523	65.073
310	23.191	69.958
330	23.419	74.891
350	19.662	79.032
370	13.926	81.966
390	10.141	84.102
410	8.740	85.943
430	8.150	87.660
450	7.519	89.243
470	6.932	90.704
490	6.259	92.022
510	5.202	93.118
530	3.923	93.944
550	2.816	94.537
570	2.053	94.970
590	1.599	95.306
610	1.351	95.591
630	1.187	95.841
650	1.026	96.057
670	0.861	96.238
690	0.731	96.392

Bin Centre (nm)	Concentration (E6 particles/ml)	Percentile Undersize (%)
710	0.669	96.533
730	0.669	96.674
750	0.694	96.820
770	0.700	96.968
790	0.662	97.107
810	0.585	97.231
830	0.494	97.335
850	0.416	97.422
870	0.371	97.500
890	0.364	97.577
910	0.393	97.660
930	0.445	97.753
950	0.510	97.861
970	0.573	97.982
990	0.622	98.113
1000-2000	8.959	100.000

Results

Mean:	**286 nm**
Mode:	**191 nm**
SD:	**186 nm**
D10:	**130 nm**
D50:	**235 nm**
D90:	**470 nm**
User Lines:	**0 nm, 0 nm**
Concentration:	**4.75 E8 particles/ml**
Completed Tracks:	**1200**

Measurement Conditions

Temperature:	22.80 ^{o}C
Viscosity:	0.94 cP
Frames Per Second:	30.00
Measurement Time:	60 of 60 s
Drift Velocity:	253 nm/s
Camera Shutter:	30 ms

Analysis Conditions

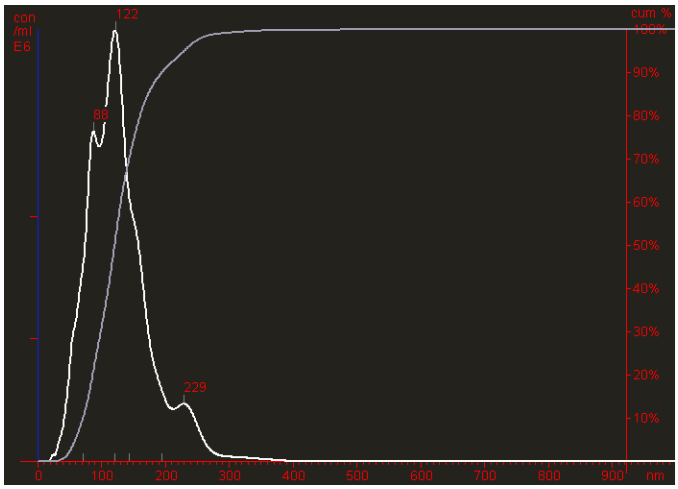
Blur:	3x3
Detection Threshold:	4 Multi
Min Track Length:	Auto
Min Expected Size:	Auto - failed

NANOSIGHT

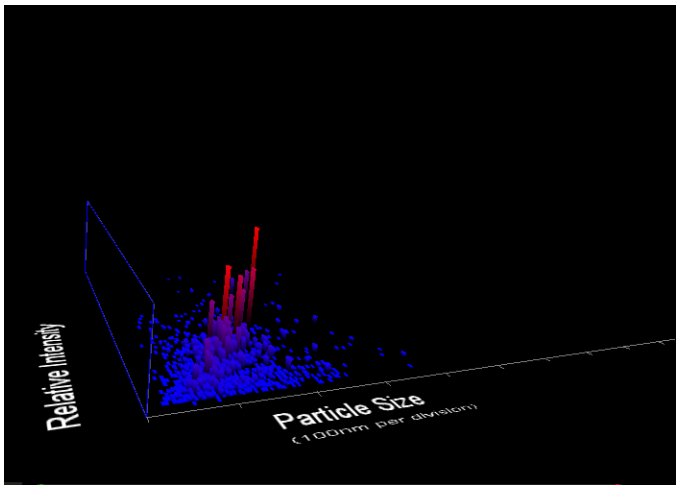
Nanoparticle Tracking Analysis (NTA) Version 2.3 Build 0025

ANALYSIS REPORT

Sample: PBCA 1:500 day19
Date/Time of Capture:
Video File: sample 102 day19.avi analysis no: 001
Operator:
Comments:



Particle Size / Concentration



Particle Size / Relative Intensity 3D plot

Bin Centre (nm)	Concentration (E6 particles/ml)	Percentile Undersize (%)
10	0.003	0.001
30	2.187	0.660
50	14.237	4.948
70	31.246	14.361
90	51.844	29.978
110	60.536	48.213
130	62.451	67.025
150	40.455	79.212
170	25.218	86.808
190	13.588	90.902
210	9.057	93.630
230	9.235	96.412
250	6.306	98.311
270	2.289	99.001
290	0.978	99.295
310	0.756	99.523
330	0.617	99.709
350	0.456	99.846
370	0.295	99.935
390	0.150	99.980
410	0.053	99.996
430	0.012	100.000
450	0.001	100.000
470	0.000	100.000
490	0.000	100.000
510	0.000	100.000
530	0.000	100.000
550	0.000	100.000
570	0.000	100.000
590	0.000	100.000
610	0.000	100.000
630	0.000	100.000
650	0.000	100.000
670	0.000	100.000
690	0.000	100.000

Bin Centre (nm)	Concentration (E6 particles/ml)	Percentile Undersize (%)
710	0.000	100.000
730	0.000	100.000
750	0.000	100.000
770	0.000	100.000
790	0.000	100.000
810	0.000	100.000
830	0.000	100.000
850	0.000	100.000
870	0.000	100.000
890	0.000	100.000
910	0.000	100.000
930	0.000	100.000
950	0.000	100.000
970	0.000	100.000
990	0.000	100.000
1000-2000	0.000	100.000

Results

Mean:	**128 nm**
Mode:	**122 nm**
SD:	**49 nm**
D10:	**72 nm**
D50:	**121 nm**
D90:	**194 nm**
User Lines:	**0 nm, 0 nm**
Concentration:	**3.32 E8 particles/ml**
Completed Tracks:	**1337**

Measurement Conditions

Temperature:	23.80 ^{o}C
Viscosity:	0.91 cP
Frames Per Second:	30.00
Measurement Time:	60 of 60 s
Drift Velocity:	404 nm/s
Camera Shutter:	30 ms

Analysis Conditions

Blur:	3x3
Detection Threshold:	4 Multi
Min Track Length:	Auto
Min Expected Size:	Auto