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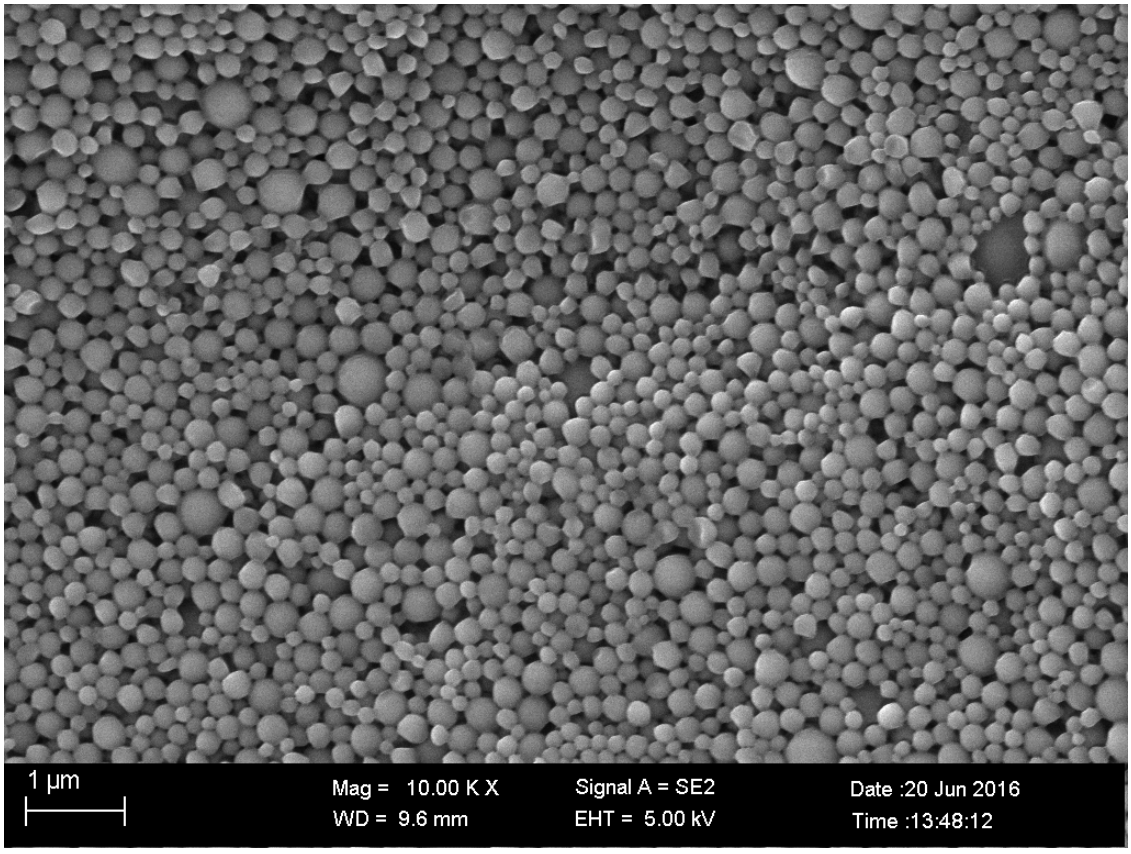

The effect of PLGA nanoparticles on the osteogenic differentiation of hMSC

A study of the synthesis of poly(lactic-co-glycolic acid) nanoparticles and how they affect induced osteogenic differentiation of human mesenchymal stem cells

Master's thesis in Materials Chemistry

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CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2017

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Abstract

The polymer poly(lactic-co-glycolic acid) (PLGA) is both biocompatible and biodegradable and has therefore become one of the most frequently used polymers in the biomedical field. In this thesis, a literature study has been conducted on PLGA as a biomaterial. PLGA nanoparticles with a diameter of approximately 400 nm have been produced with the solvent evaporation method and the synthesis process has been analyzed and optimized. The synthesized nanoparticles have been used in *in vitro* studies on human mesenchymal stem cells, where their impact on cell viability and osteogenic differentiation has been investigated. For the nanoparticle synthesis, it was found that the concentration of stabilizer had the greatest impact on the result. Increased amount of stabilizer produced smaller particles. The *in vitro* studies were difficult to interpret, but implied that cell viability was negatively affected immediately after introduction of PLGA nanoparticles but that the cells quickly recovered. No difference in amount of expressed alkaline phosphatase, an osteogenic marker, could be observed between differentiated and non-differentiated samples. Deposits of extracellular calcium, another sign of osteogenesis, seemed to be enhanced by the particles upon visual inspection. However, this could not be proven quantitatively. These findings suggest that PLGA nanoparticles have an effect on the osteogenic differentiation of hMSCs, but the nature and extent of it needs to be investigated further.

Keywords: PLGA, nanoparticles, hMSC, osteogenesis, osteogenic differentiation, solvent evaporation method.

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Thanks, xièxie, shukran and tack!

Lukas Dahlgren, Gothenburg, January 2017

Contents

1
Introduction

Poly(lactic-co-glycolic acid), abbreviated PLGA, is a polymer that has been extensively studied the last decades. The polymer is biocompatible and approved by the Food and Drug Administration (FDA) for medical use. It is biodegradable and the degradation time can be controlled, H.K. Makadia (2011). PLGA can be formed into almost any shape and the surface can be easily modified, Jiang et al. (2015). The many beneficial properties of PLGA have attract a lot of attention to the polymer in the fields of drug delivery and tissue engineering.

Surprisingly few articles have investigated pure PLGA particles, alone and free from coatings and grafts. In fact, only one article to my knowledge has investigated how nanoparticles of PLGA affect the differentiation of the cells, Jiang et al. (2015). The authors covered PLGA nanoparticles in bovine serum albumin and studied their interaction with mesenchymal stem cells from rats. Their findings showed that PLGA nanoparticles might induce osteogenesis.

My thesis expand on this and has investigated how to synthesize non-coated PLGA nanoparticles and how these particles affect the differentiation of human mesenchymal stem cells. The results could improve the understanding of how PLGA nanoparticles interact with human cells as well as pave the way for new approaches in tissue engineering.

This thesis is divided in two parts. The first one is a literature study of PLGA as a biomaterial. It covers the properties of the polymer and how it behaves as a nanoparticle. The literature study also includes theory of different synthesis methods for PLGA nanoparticles and how the synthesis parameters are expected to affect the result. A brief summary of existing literature on PLGA in the field of bone tissue engineering is also included together with information concerning human mesenchymal stem cells. The literature study ends with a theoretical background of the analysis methods used in this work. The second part of the thesis covers the practical work. The aim for this part was to synthesize nanoparticles of PLGA, optimize this procedure and finally investigate how the particles affect the viability and osteogenic differentiation of human mesenchymal stem cells.

This study is a part of the project *Influence of nanoparticles on the differentiation of mesenchymal stem cells* conducted by Q.L Feng's research group at Tsinghua University. The particle synthesis was performed in collaboration with Simon Myrbäck, Myrbäck (2017).

2
Theory

This chapter is mainly a literature study of poly(lactic-co-glycolic-acid) as a biomaterial. The chemical properties of the polymer are explained and how the material interacts in a biological system, with special focus on biocompatibility, degradation and drug release. The chapter also covers the use of nanoparticles in medicine, both in general and when made of PLGA. This section depicts how PLGA nanoparticles are utilized in medicine, advantages contra disadvantages with the material and sterilization techniques. In addition the section explains different methods of synthesizing PLGA nanoparticles and how the synthesis parameters influence the result. The next section contains a summary of existing research on PLGA in bone generation (osteogenesis) followed by some information about human mesenchymal stem cells. Lastly comes a section where all the methods for sample analyzation used in this work are explained briefly.

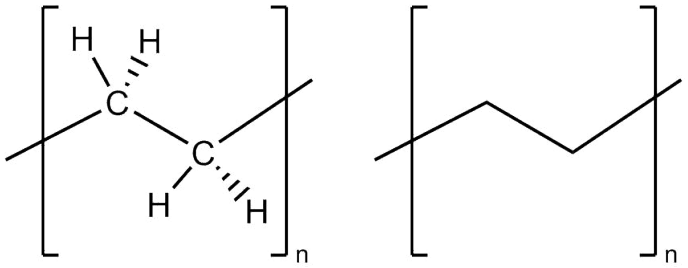
2.1 Poly(lactic-*co*-glycolic acid)

Poly(lactic-*co*-glycolic-acid), henceforth abbreviated PLGA, is a synthetic copolymer that has attracted a lot of attention in the fields of drug delivery and tissue engineering the last decades. The polymer possesses some very desirable properties, mainly biocompatibility and biodegradation. PLGA is approved by Food and Drug Administration (FDA) for several medical applications and is generally considered to be non-toxic, Sharma et al. (2011). PLGA degrades fast in the body compared to other synthetic polymers and the residues are transformed in the citric acid cycle to water and carbon dioxide. The degradation time, mechanical properties and drug release rate are all tunable and PLGA can be formed in a wide range of shapes and sizes, H.K. Makadia (2011) Another advantage of PLGA is that the polymer has been studied extensively for a long time and a lot of knowledge of synthesis methods and medical effects is therefore available.

2.1.1 An introduction to polymers

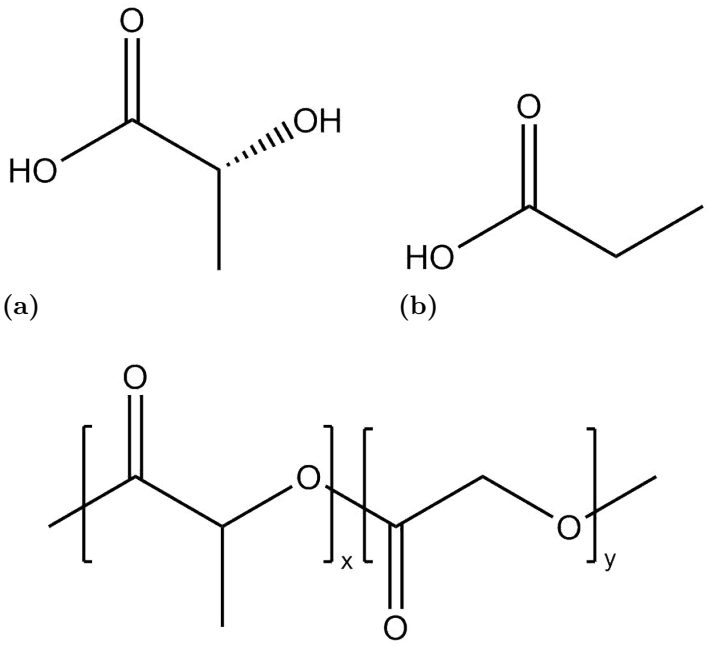
A polymer is a macromolecule that consists of several repeating units called mers. A common misconception is that a mer is the same as a monomer, where in fact a monomer is the starting molecule that becomes a mer after polymerisation. An example of a simple polymer is polyethene which is just a carbon chain, see figure 2.1. Here the monomers are ethene molecules (C_2H_4) while the mers lack the double

bond. A copolymer is made from two or more different types of monomers. The monomers of PLGA are lactic acid and glycolic acid, see figure 2.2 (a) and (b), and the complete copolymer is seen in figure 2.2 (c). The ratio between the monomers can vary and often has a big impact on the properties of the copolymer, see section 2.1.4 for more information.



(a) **(b)**

Figure 2.1: (a) The mer of polyethylene (b) Same mer, but more efficiently depicted



(a) **(b)**

(c)

Figure 2.2: (a) Lactic acid (b) Glycolic acid (c) Poly(lactic-*co*-glycolic-acid), where x and y depict the number of mers of lactic acid and glycolic acid respectively.)

Polymers are often divided into two classes; naturally derived and synthetic polymers. Natural polymers can, as the name implies, be found in nature. Examples of natural polymers are proteins like collagen or carbohydrates like cellulose. Both these polymers are potential biomaterials. Other natural polymers that is being researched as candidates for biomaterials are chitosan, found in the exoskeletons

of arthropods; agarose, formed by algae and alginate which is derived from seaweed, J.S. Temenoff (2008). Synthetic polymers are man-made, for example PET, nylon and Kevlar. Even though many synthetic polymers are used as biomaterials, like PHEMA (poly(2-hydroxyethyl methacrylate)) for contact lenses and PDMS (polydimethylsiloxane) for breast implants, J.S. Temenoff (2008), only a few of the biodegradable ones are approved by the U.S. Food and Drug Administration (FDA). PLGA is one of them together with its monomers polyglycolic acid and polylactic acid, J.S. Temenoff (2008).

Whether to use natural or synthetic polymers depends on the application and the polymer. Each case is different, but a few guidelines exists. Natural polymers are often similar to the tissue they interact with and may therefore integrate easier. They are also derived from renewable resources. Synthetic polymers on the other hand seldom interact with the tissue and are often (but not always) produced from non-renewable resources. They can however be mass produced and sterilised, thus providing a steady supply and minimize the risk for phatogens. The biggest advantage of synthetic polymers is the control of the properties. Mechanical, chemical and physical properties can all be tailored, J.S. Temenoff (2008).

2.1.2 Stereochemistry

Lactic acid is a chiral molecule containing an asymmetric α carbon. The two enantiomers, the dextrorotatory (D) form and the levorotatory (L) (not to be confused with the prefixes rectus (R) and sinister (S) which refers to the configuration of a single stereocenter and not the whole molecule), are displayed in figure 2.3. Note that the two forms cannot be superposed. In PLGA the two enantiomers are generally present in in equal ratio, H.K. Makadia (2011). Glycolic acid is an achiral molecule and therefore lacks enantiomers.

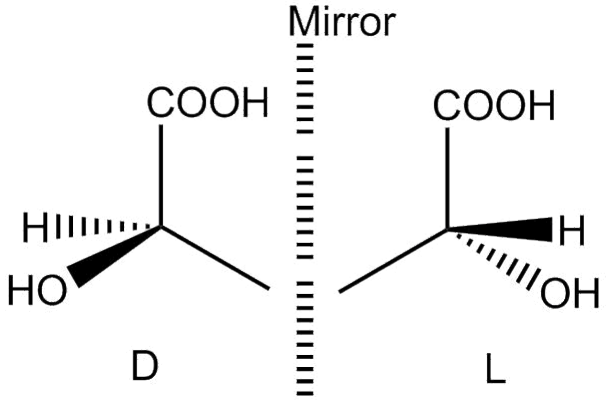


Figure 2.3: The two enantiomers of lactic acid, dextrorotatory (D) to the left and levorotatory (L) to the right.

2.1.3 Biocompatibility

PLGA is, as mentioned before, one of the few synthetic polymer that has been approved by regulatory agencies for clinical use in humans, J.S. Temenoff (2008). Most if not all articles studying the polymer as a biomaterial state its biocompatibility as the biggest advantage. But before delving further into this topic, it is important to understand the definition of biocompatibility.

Contrary to popular belief, no material can be said to be truly biocompatible. A material is only deemed biocompatible after rigorous evaluation in a specific application. The International Union of Pure and Applied Chemistry's (IUPAC) definition of biocompatibility, which is the one J.S. Temenoff (2008) uses, reads as follows:

"Ability of a material to perform with an appropriate host response in a specific application"

With this follows that whenever a material is claimed to be biocompatible the intended application must be stated as well. Titanium for example, famous for being biocompatible, makes poor capsules for drug delivery since it does not degrade. Polyurethanes are found in artificial blood vessels but lack the mechanical strength to be used as bone implants. Even the expected host response should be stated. Biomaterials often comes with some implications and it is important to ensure that the benefits outweigh the risks.

Even though the definition addresses materials, regulatory agencies do not. In order to launch a product on the market the complete device must first have approval of a suiting agency, like Food and Drug Administration (FDA) in the U.S or European Medicines Agency (EMA) in Europe. It is not enough that the product is made of a material that has been considered biocompatible in the past, J.S. Temenoff (2008).

FDA has become the general guideline since the U.S. is a big market and because the agency is well renown. A FDA-approval therefore facilitates approval from other regulatory agencies. The path to get a FDA approval is often a long procedure, especially for completely new products. Generally the evaluation includes the following four stages, J.S. Temenoff (2008):

In vitro testing.
In vivo studies with healthy experimental animals
In vivo studies with animal models of disease (if applicable)
Controlled clincal trials

The amount of tests required is decided by the class of the device. FDA classifies all medical devices into three categories where class I poses low risk (for example dental floss) and is therefore subject to the least regulatory controls while devices in class III (for example heart valves replacements) possess the highest risk of affecting the patient's health and are therefore subject to the highest level of regulatory control, U.S. Food and Drug Administration (2015). If a similar product already exists on

the market it could be sufficient to obtain a so called 510(k) classification, which is indeed the most common pathway to the market for medical devices, Anusavice et al. (2013). A review is conducted to determine whether the device shows *substantial equivalence* (SE) with an already existing product on the market. If the device is deemed at least as safe as an existing product on the market it can get a 510(k) classification and be launched. If not, then a *Premarket approval* (PMA) could be relevant. A PMA is required when not enough information is known about the safety and efficiency of a new device and includes the most thorough testing. Class III devices are usually required to have a PMA before launch, Anusavice et al. (2013).

PLGA is generally considered to be biocompatible because of its degradation products i.e the monomers occur naturally in the body. Lactic acid is most well known to accumulate in the muscles during intensive exercise. Its conjugated base, lactate is produced during the glycolysis which is the anerobic process of oxidizing glucose into pyruvate. During the oxidation the high energy carrier ATP (adenosine triphosphate) is formed from ADP (adenosine diphosphate) and NAD^+ (dicotinamide adenine dinucleotide) is reduced and forms the the electron carrier NADH. NAD^+ can be regenerated by reducing pyruvate and the glycolisis can continue, Alberts (2008). If oxygen is accessible lactate will oxidize back to pyruvate. The pyruvate will be transported from the cytosol where the glycolysis occured to the mithochondria, where the enzymes in the pyruvate dehydrogenase complex breaks it down to CO_2, NADH and acetyl CoA (Coenzyme A), Alberts (2008). Acetyl CoA is then oxidized in the citric acid circle. The citric acid circle, also known as the tricarboxylic acid cycle or Kerbs cycle, is a series of reactions that produce energy from acetyl CoA, which in turn could be derived from sugars as mentioned but also from fatty acids and proteins. The major end products after the oxidization are CO_2 and NADH. NADH is used in the electron transport chain to generate ATP and the protons are combined with respiratory oxygen to obtain water as a waste product, Alberts (2008). This is why it is legitimate to say that lactic acid turns into CO_2 and H_2O, Gunatillake & Adhikari (2003), even though it is a simplification. It is often suggested that glycolic acid takes the same route, Sharma et al. (2011), Lü et al. (2014), Danhier et al. (2012). It has also been proposed that glycolic acid can in addition pass through the kidneys and be excreted with the urine, Ducheyne et al. (2015), or broken down by enzymes, especially those with esterase activity, Gunatillake & Adhikari (2003). Hollinger (1983) proposed that glycolic acid does not go through the citric acid cycle at all but is enzymatically converted to glyoxylate by glycolate oxidase and then turned into the amino acid glycine by glycine transaminase.

When synthesizing PLGA the levorotatory enantiomer of lactic acid, LLA, is often the choice since it is the form that occurs naturally in the body and is preferentially metabolized, Gunatillake & Adhikari (2003), Sharma et al. (2011), Alexis (2005). Even so, no studies have showed any adverse effects when using PLGA containing both enantiomeric forms to my knowledge.

Many *in vitro* and *in vivo* studies have showed that PLGA is sufficiently biocompatible, Ducheyne et al. (2015), although some studies carried out in the early 90s

suggest otherwise. One known concern is when the polymer is used in big implants, such as in orthopaedic applications. The large amount of acidic degradation residues can result in local areas with very low pH, Gunatillake & Adhikari (2003). The same effect is not experienced with smaller devices, such as micro- and nanospheres.

Biodegradable sutures of PLGA got FDA-approval during the end of the 1970s and were launched at the marketed as Vicryl and Polyglactin 910, Ducheyne et al. (2015). This approval has made it easier for other PLGA-based medical devices to reach the market and is one of the reasons why PLGA is the preferred synthetic polymer for these applications. Since then several other devices have gotten approval, microparticles, implants, gels to name a few. A summary of the FDA-approved PLA (polylactic acid) and PLGA-based drug products available on the U.S. market in June, 2016 can be seen in figure C.1 in appendix C.

2.1.4 Degradation

Perhaps the most interesting feature of PLGA is that the material degrades in the body, a trait henceforth referred to as biodegradation. Before venturing further on this matter the terminology ought to be explained. In this work the definition of biodegradation follows the one presented in J.S. Temenoff (2008):

"*...biodegradation as the chemical breakdown of a material mediated by any physiological environment.*"

Put in other words, biodegradation is the breakage of chemical bonds of a material inside a living organism. Important to note here is that although the prefix "bio" normally requires the process to carried out by a biological entity, for example cells, bacteria or proteins, biodegradation occurs in a physiological environment which also include abiotic factors like water, pH and ions. This is especially significant for PLGA which is hygroscopic, meaning that the polymer attracts water.

PLGA degrades mainly by hydrolysis, which is the name of the process where water molecules facilitate the cleavage of chemical bonds in a macromolecule, J.S. Temenoff (2008). The meres of PLGA are linked to each other by ester bonds, a linkage which is susceptible to hydrolysis. The biodegradation of PLGA can be explained in four consecutive steps: hydration, initial degradation, further degradation and solubilization, Wu & Wang (2001). The water molecules disrupt the van der Waals forces and hydrogen bonds between the polymer chains and thus act as plasticizers. This lowers the glass transition temperature (Tg, explained in section 2.2.3) and relaxes the polymer. In the second step, degradation starts with hydrolysis of the polymer backbone. The molecular weight decreases and the polymer loses its mechanical properties but maintains its integrity and most of its mass. Further degradation occurs when a critical molecular weight is reached and the polymer can not hold its integrity. Mass loss follows since oligomers (molecules with few mers) start to diffuse out. Water molecules fill the void which speeds up the degradation, Engineer et al. (2011). In the final stage the polymer matrix has become highly porous

and hydrated which results in homogeneous and slower degradation, Engineer et al. (2011). The oligomers are further cleaved into soluble molecules, Wu & Wang (2001).

Hydrolytic degradation in polymers can be divided into two types, bulk and surface degradation. In bulk degradation, water penetrates the polymer faster than the degradation rate causing a rapid decrease in mechanical properties even though the original shape and size is mostly maintained. Surface degradation is the opposite scenario, where the rate of the polymer hydrolysis is faster than the penetration of water. This causes the material to shrink in size but its mechanical properties are maintained, J.S. Temenoff (2008). PLGA is often considered to undergo bulk degradation, H.K. Makadia (2011), Gentile et al. (2014), Alexis (2005). Anderson & Shive (2012) and Duan et al. (2007) claim that PLGA microparticles smaller than 300 μm undergo homogeneous degradation where the degradation rates of the surface and the core are equal, although the article they both refer to, Spenlehauer et al. (1989), does not investigate size dependency and seems to state the opposite as the following can be read in Spenlehauer et al. (1989): "*SEM analysis of the microspheres during in vitro degradation showed that the surface of microspheres is degraded more slowly than the core.*". Homogeneous degradation should therefore be deprecated until more reliable sources can be presented.

Control is key when utilizing biodegradation. Uncontrolled degradation is almost always undesired. Degradation changes the properties of the biomaterial and could result in poisonous residues. An example of an area where degradation is highly undesired is hip replacement since it is crucial that the implant maintains its properties and shape in order to function satisfactory. Examples of applications which utilise biodegradation are drug delivery and scaffolds for tissue engineering. In these areas it is important to have a thorough understanding of degradation rate, how it affects the properties of the material and what residues it creates. The rate and extent of the biodegradation of PLGA depend on several factors. Here follows an overview of most of them. These factors can be inherent to the polymer itself or external, but they are all connected to how available the ester bonds are to water molecules.

Crystallinity
The structural order of the polymer chains affects the properties of the material. A polymer is crystalline when the chains are folded in an ordered fashion. When the opposite is the case, when the structure is random, the material is amorphous. Factors that determines the crystallinity are for example intermolecular bonds (hydrogen bonds, polarity and charge), hydrophobic interaction and steric hindrance.

The monomers of PLGA have different degrees of crystallinity. The methyl group of lactic acid, henceforth abbreviated LA, is the only difference in chemical structure between it and glycolic acid, henceforth abbreviated GA, but it has a high impact on the degree of crystallinity. GA is highly crystalline while the methyl group of LA introduces a steric hindrance which obstructs efficient packing and thus decreases crystallinity, Gentile et al. (2014). LA can be made crystalline if only one of the enantiomers is used, and the degree of crystallinity can be varied by changing the

ratio of D and L forms. PDLA and PLLA can pack more easily compared to the racemic PDLLA and thus have a higher degree of crystallinity.

The degree of crystallinity of the copolymer PLGA is affected by the types of enantiomer used and the the molar ratio between the two, Ducheyne et al. (2015). Racemic PLGA is often amorphous while PLLGA can be made semi crystalline, Ducheyne et al. (2015). PLGA starts to crystallize with a GA content of approximately 70% and above, Ducheyne et al. (2015), Gilding & Reed (1979). Crystallization also occurs when the GA content is lower than 20% and LA is non-racemic, Gilding & Reed (1979). PLGA is amorphous in that interval. Other factors that decide the degree of crystallinity are molecular weight, H.K. Makadia (2011), and synthesis method, Alexis (2005).

The degree of crystallinity affects attributes such as mechanical strength, swelling behavior and capacity to undergo hydrolysis which all have a direct impact on the biodegradation rate. The relationship between crystallinity and degradation rate is a bit unclear however. There are some conflicting reports on the matter, H.K. Makadia (2011), Alexis (2005). The contradicting results have been attributed to the difficulties to compare the reports. The studies differs in that they have used different synthesis methods, drug loadings and PLA instead of PLGA. However, the general consensus seems to be that amorphous (low or zero degree of crystallinity) PLGA degrades faster than PLGA of higher degrees of crystallinity, Dinarvand et al. (2011), Thakur & Thakur (2015). The explanation is that the polymer chains of ordered crystalline regions are less accessible for water molecules than loose chains in an amorphous material and are therefore less susceptible to degradation through hydrolysis.

An interesting behavior of PLGA polymers is that the crystallinity increases during degradation, Engineer et al. (2011). This could be attributed to two factors, the first being that the amorphous regions degrades first thus leaving the crystalline regions left. The other factor could be that GA is degraded first which increase the portion of LA and thus facilitates crystallization.

Mer ratio
The ratio between lactic acid and glycolic acid affects the degradation rate. The molecular difference between the two monomers is just the occurrence of an extra methyl group in lactic acid, but it is enough to make it more hydrophobic and thus less susceptible to water. Therefore the degradation rate of PLGA is increased with a higher GA content, with the exception of 50:50 composition which exhibits the fastest degradation, Gentile et al. (2014). It is difficult to find literature investigating PLGA consisting of mainly GA. Park (1995) makes a thorough investigation of the effect of copolymer composition on the degradation rate but leaves out polymers with high GA content. A fairly old article, R.A. Miller (1977), and Gentile et al. (2014) do test PLGA with the compositions 25:75 and 10:90 respectively, but the results are hard to interpret. However, the general consensus remains that the 50:50 composition has the fastest degradation rate because it experience the lowest crys-

tallinity, Gilding & Reed (1979). Generally the degradation rate increases with a higher content of GA, with the exception of 50:50, but at 70% GA the water content starts to drop due to increased crystallinity, Gilding & Reed (1979).

Tacticity
Even though PLGA is always depicted as having alternating mers of lactic acid and glycolic acid the composition is more random in reality. It is not completely random though because monomers of the same type tend to be incorporated next to each other in the polymer chain, thus creating block of varying length consisting of the same mer, H. Qian (2011). This affect the physical properties of the material because PLGA with random sequence degrades quicker than alternating, Gentile et al. (2014). The meres of glycolic acid lack a methyl group compared to lactic acid which make glycolic rich areas of the polymer chain more susceptible to water which degrades the polymer chain through hydrolysis, C.A.C Erbetta (2012). For alternating PLGA the molecular weight loss profile is almost linear throughout the hydrolysis, which is in stark contrast to a random copolymer with the same monomer ratio, Li et al. (2011). Li et al. (2011) and H. Qian (2011) among others have suggested methods to increase the control of the polymerisation.

Since LA is chiral it is possible to produce PLGA with different stereochemistry. The copolymer will have the same form as the monomers used to synthesise it, meaning that if the stereo-regular forms of pure DLA or pure LLA is used the end result will be PDLGA or PLLGA respectively, Ducheyne et al. (2015). Racemic PLGA, which is a mixture of both D and L in equal ratio, is most frequently used, H.K. Makadia (2011), because it has a higher degradation rate, Li et al. (2011). A racemic mixture is more amorphous since the combination of two different enantiomers makes crystallisation difficult, which in turn enables water molecules to reach the ester bond of the polymer background and degrade them through hydrolysis.

Molecular weight
The molecular weight of a polymer can in brief be explained as the atomic mass of each polymer chain, i.e. the size of a individual chain. Molecular weight is one of the major factors affecting the degradation rate according to several reviews on the topic, Gentile et al. (2014), H.K. Makadia (2011), Alexis (2005). The very same reviews also imply that an increased molecular weight results in a lower degradation rate. However it is hard to find evidence of this when digging deeper. In the case of H.K. Makadia (2011) the review states vaguely that "*Polymers with higher molecular weight have generally exhibited lower degradation rates*" which may be true but is not specific for PLGA. Gentile et al. (2014) boldly declares that "*increasing the molecular weight of conventional PLGA from 10–20 to 100 kDa, degradation rates were reported to range from several weeks to several months*" without referring to any article. Alexis (2005) referrers to one study (Wang N (1998)) where it was shown that PLGA with a molecular weight of 3.025 kDa had a slower degradation rate compared to a sample of 1.317 kDa. It is worth mentioning that both samples had relatively small molecular weights and were even referred to as oligomers by the authors instead of polymers. Alexis (2005) continues with referring to another study

(Wu & Wang (2001)) which states the opposite. Samples with molecular weight of 11, 31, 67, 125 and 167 kDa were investigated in this article with the clear conclusion that the degradation rate is increased with a higher molecular weight. From these two conflicting studies Alexis (2005) makes the conclusion "*In summary, it is clear that the lower the molecular weight, the faster the degradation and drug release.*". But it does not stop there. The articles Lanao et al. (2011) states "*Considering molecular weight, high molecular weight polymers are known to degrade in a slower ratio than low molecular weight polymers*" and referrers to two articles, one of which investigates PLA instead of PLGA while the other one is Wu & Wang (2001) which as mentioned earlier states the complete opposite relationship between molecular weight and degradation rate.

Worth noting is that it often is hard to compare different studies since they seldom are executed in the same manner. The size of the sample, degradation medium and testing time could all affect the result. My own conclusion, based on the work of Wu & Wang (2001) and Li et al. (2011), is that high molecular weight PLGA indeed expresses a faster degradation rate, at least initially. A theory of why is that longer polymer chains has more sites susceptible for hydrolysis. The effect of weight loss is also much more pronounced in polymers with high initial molecular weight, for example a 50% loss can be expected to change the physical properties of a polymer with an initial molecular weight of 100 kDa much more than one with of only 1 kDa. I believe another reason for the confusion is that concepts are mixed up. Authors are talking about PLA like it was PLGA, degradation could be measured in different ways and molecular weight has several definitions, mass average molecular weight and number average molecular weight being two. The biggest misconception in my opinion is when degradation rate and total degradation are interchanged. An example is H.K. Makadia (2011), where one can read "*Polymers having higher molecular weight have longer polymer chains, which require more time to degrade than small polymer chains*". This statement is not in conflict with the theory that higher molecular weight results faster degradation rates. Since degradation often is defined as loss of molecular weight, polymers with a high initial molecular weight can express a high degradation rate and thus lose a high percentage and still have a higher molecular weight than polymers with a low initial molecular weight. In other words, polymers with a high molecular weight can both possess a higher degradation rate and longer degradation times. This seems to be the case with PLGA.

Carboxylic end groups
When the polymer chain of PLGA is cut (a process called chain scission) during degradation carboxylic end groups are formed. These speed up the polymer chain scission through a process called autocatalysis, where the products also act as reactants in the hydrolysis, Wu & Wang (2001). This might be an explanation of why PLGA materials generally display bulk degradation. The degradation residues with carboxylic endgroups diffuse away at the surface while they remain in the bulk and can participate in the hydrolysis, Alexis (2005), Ducheyne et al. (2015). Studies by Tracy et al. (1999) and Lanao et al. (2011) showed that the degradation rate could be tuned by modifying the end groups of the polymer. PLGA has normally acidic

caboxyl groups at the polymer terminus. So called capped PLGA has instead an ester linkage at the terminus, Lanao et al. (2011). One study, Tracy et al. (1999), showed that uncapped PLGA degrades 3-4 times faster and both studies, Tracy et al. (1999) and Lanao et al. (2011), came to the conclusion that the type of end groups has a bigger impact on the degradation rate compared to initial molecular weight. The carboxyl groups are more hydrophilic and therefore get hydrated faster. The acidic end groups also catalyse the hydrolysis, Tracy et al. (1999).

External factors
External factors affect the degradation as well. The molecular weight loss of PLGA is almost unchanged when stored at 5 °C while while it drops rapidly at temperatures of 37 °C and above, Ducheyne et al. (2015). The glass transition temperature, the temperature at which the polymer chains start to move independently from each other and the material becomes less rigid and more glassy, of PLGA is reported to be around 37 °C and above, Gentile et al. (2014), and this extra movement facilitates hydration.

pH affects the degradation rate as well. Wu & Wang (2001) compared degradation rate of PLGA in pH 5 (acidic), pH 7.4 (neutral) and pH 9.24 (basic). No difference could be observed during the first two weeks, probably because the samples got hydrated during this time. However, during the third week the degradation of the basic sample started to slow down and soon reached a plateau where the molecular weight remained unchanged. The other samples continue to drop in molecular weight with the acidic sample exhibiting the fastest degradation. This behavior is attributed to the collapse of the polymer's integrity at a certain point during the degradation. Hydroxides (OH^-) can then diffuse freely inside the polymer matrix and neutralise the caboxylic groups of the degradation residues and therefore impair the autocatalysis. The opposite happens in the acidic sample, where hydroniums (H_3O^+) catalyze the hydrolysis reaction.

During degradation the pH is reduced due to the acidic end groups of the hydrolysis products and reaches values of pH 2, Wu & Wang (2001). In microparticles of PLGA, the pH becomes lower in the center of the particles compared to the surface because of the autocatalytic activity of degradation products, Ducheyne et al. (2015). This effect becomes less pronounced when the size of the particles decreases since the degradation products diffuse faster out of the particle's interior, Fu et al. (2000). This is important to consider when PLGA particles are used to deliver pH-sensitive drugs, and nanoparticles might be an option in these cases.

There is an ongoing debate whether PLGA can be enzymatically degraded or not, H.K. Makadia (2011), Alexis (2005). Some studies suggest this but so far the data has been conflicting, Alexis (2005), and not convincing, Anderson & Shive (2012). The main issue is the lack of standardized *in vivo* tests which makes it difficult to compare the different experiments, H.K. Makadia (2011), Alexis (2005). The results by Tracy et al. (1999) among others show that PLGA degradation occurs faster *in vivo* compared to *in vitro*. Enzymes are mentioned as a plausible explanation to-

gether with other molecules, like free radicals and acidic products, produced by cells during the foreign body response. Another explanation is the presence of lipids and other hydrophobic biomolecules that act as plasticizers and allow for higher mobility for the polymer chain which facilitates hydration. Proteinase K has a role in the degradation of PLA since it has been proven that it preferentially degrades L-lactyl units (lactyl is a derivative of lactic acid) compared to D-lactyl units, Alexis (2005), which could have an impact of the choice of enantiomer during the synthesis. Similar experiments with PLGA instead of PLA have not been conducted to my knowledge. Worth noting is that enzymatic degradation is troublesome from a medical point of view since each patient has an unique physiology. Degradation through hydrolysis on the other hand is universal.

2.1.5 Drug release

One of the major application of PLGA in medicine is in the field of drug delivery, H.K. Makadia (2011). The major advantage of micro- and nanoparticles of PLGA for encapsulation of therapeutic drugs, besides biocompatibility, drug protection and ease of administration via injection, is the ability to have an extended release which can be tuned to be days, weeks and even months, Mundargi et al. (2008). It has also been proved that PLGA devices are excellent vectors for biomolecules such as nucleic acids, proteins and vitamins, Sharma et al. (2011). But to fully exploit controlled release the kinetics need to be understood.

Naturally, drug release is highly connected to particle degradation and the variables involved are just as many. This offers a lot of flexibility but makes exact release pattern difficult to predict. On top of that the release profile of PLGA devices is seldom of linear zero order which is preferable since it facilitates calculations, Sharma et al. (2011). The release pattern for PLGA biodegradation seems to follow a biphasic or sometimes triphasic pattern, H.K. Makadia (2011), Sharma et al. (2011), Mundargi et al. (2008), with an initial rapid burst of drug release followed by a phase with a slower release rate. When the device comes into contact with a water-based medium it will quickly swell because of water penetration and diffusion will become significant. Drugs present on the surface as well as drugs in walls of the newly created pores will be released through diffusion as a function of solubility, Sharma et al. (2011), H.K. Makadia (2011). After the initial burst the release pattern is govern by the degradation of the polymer matrix itself, Dinarvand et al. (2011).

It seems to be some confusion in the scientific world on the topic of why PLGA exhibits release kinetics of non-zero order. Some studies claim it is because PLGA degrades homogeneously, Alexis (2005), H.K. Makadia (2011), while others state the reason is heterogeneous degradation, Gentile et al. (2014), Sharma et al. (2011), Engineer et al. (2011). The reason for this contradiction is probably rushed conclusions of the mechanisms behind the degradation. As mentioned earlier the general consensus is that PLGA devices undergo bulk degradation, which often but not always equals homogeneous degradation i.e. the bulk degrades as fast as the sur-

face. Zero order kinetics is generally exhibited by materials that undergo surface degradation. Therefore a premature conclusion could be that PLGA degrades homogeneously since it undergoes bulk degradation and does not exhibit zero order kinetic for drug release. However, in the case of PLGA devices, an autocatalytic degradation occurs in the bulk, see section 2.1.4. The acidic degradation residues diffuse away on the surface while they remain trapped in the bulk, thus accelerating the degradation rate in the bulk of the material, Engineer et al. (2010). Therefore PLGA devices degrade heterogeneously but with faster rate in the bulk compared to the surface and not the other way around. This explains why the material displays release kinetics of non-zero order even though it degrades heterogeneously.

Following part expands more of the process of drug delivery and is entirely from Sharma et al. (2011) unless otherwise noted.
Drug molecules are said to be released in three ways:

I. Transport through pores
II. Transport through the polymer matrix
III. Dissolution of encapsulating polymer

PLGA is hygroscopic due to the hydrophilic ester bonds and a flexible polymer backbone chain. Devices made of the polymer will therefore absorb water and swell. Small pores will form which will grow in size and connect to form a network. Encapsulated drugs, especially hydrophilic ones, will diffuse out through the pores due to the chemical potential gradient. A different form of transport is convection through osmosis where the driving force is a solute gradient over a semi-permeable membrane, a membrane which allows transport of the pure fluid but not the solute. The polymer matrix can under certain circumstances act as a semi-permeable membrane and the osmotic pressure is then govern by the solute concentration of the encapsulated drug and the surrounding medium. The release profile is directly related to the degradation and is affected by virtually the same parameters such as molecular weight, composition, fabrication methods etc. The type of drug matters as well as it can affect the degradation rate and therefore also the release profile, Engineer et al. (2011).

2.2 Nanoparticles

Nanotechnology is a broad field of science which studies materials with at least one dimension in the nanoscale. The range of this nanoscale varies somewhat depending on who you ask. A common definition is between 1-100 nm (nanometers = 10^{-9} meters), Cademartiri & Ozin (2009) but there are others. Dinarvand et al. (2011) defines nanoparticles for pharmaceutical purposes to range in size from 10-400 nm in diameter while Mundargi et al. (2008) uses the wider interval of 10-1000 nm. In this work all particles with a submicron diameter (less than 1000 nm = 1 μm) will be referred to as nanoparticles. This work will also focus solely on spherical nanoparticles, even though interesting research is being conducted on other geometrical forms for PLGA as well, like 2D nanofilms or 1D nanofibers, Ducheyne et al. (2015). Scaffolds for tissue engineering is also a promising field.

2.2.1 PLGA nanoparticles in drug delivery

Nanoparticles of PLGA have many advantages in mainly drug delivery compared to other materials, sizes and use of pure drug. PLGA as a material is generally considered biocompatible, has been investigated extensively for a long time and is one of the few synthetic polymers that have gotten FDA-approval for drug delivery, J.S. Temenoff (2008). It has also the advantage of sustained release compared with natural polymers that generally have a relatively short duration of drug release, Lü et al. (2014). The subcellular size of nanoparticles gives them the ability to penetrate deep through fine capillaries, avoid rapid clearance by phagocytes and enter cells, Mundargi et al. (2008). Encapsulation of drugs allows for protection from degradation (enzymatic, cellular and/or environmental), sustained release and enhanced permeability and retention, Lü et al. (2014).

PLGA nanoparticles can act as a vector for transmission of not only drugs but macromolecules such as proteins, peptides, genes, vaccines, antigens, human growth factors etc, Mundargi et al. (2008). These substances can be delivered either indirectly or targeted. Targeted drug delivery is an interesting field and can be divided into two categories, passive and active targeting. Passive targeting might be utilised in cancer treatment due to the pathophysiology of the tumor vasculature, Lü et al. (2014). In short, the vasculature of tumors is more permeable and has impaired lymphatic drainage compared to healthy tissue. Macromolecules and nanoparticles will thus accumulate and maintain in the tumors, an effect known as Enhanced Permeation and Retention, Dinarvand et al. (2011). Active targeting differs in that it involves moieties that interact specifically to certain type of cells, for example neoplastic cells. These targeting agents are grafted, adsorbed or otherwise associated with the nanoparticles to allow for a more efficient and specific delivery of the drug, Lü et al. (2014). Three main categories of targeting agents exists; ligands, antibodies and aptamers, the last one being oligonucleotides of DNA or RNA which work as antibodies by binding to target antigen with high affinity and specificity, Dinarvand et al. (2011). Targeted drug delivery offers many advantages. Apart from chances of being more effective, the drug will also be more efficient since it only acts where it meant to. The drug dosage can therefore be reduced which has both economical and medical benefits since the drug itself might be both expensive and harmful, Lü et al. (2014).

Even though PLGA nanoparticles have many advantages and are expected to gain a lot of attention in the future there are some concerns that need to be considered, Sharma et al. (2011), Danhier et al. (2012) Mundargi et al. (2008):

- The interior of the PLGA nanoparticles becomes acidic due to the carboxylic end-groups of the polymer backbone, a phenomenon that could affect the activity of pH-sensitive drugs such as most proteins. See section 2.1.4 for more information. The conditions of the particles synthesis could affect the stability of the drug as well.
- PLGA nanoparticles generally displays a high encapsulations efficiency around 60 to 70%, meaning that most of the drug used for the formulation indeed ends up in the particles. However they often exhibit poor drug loading, which is the

ratio between drug and particles. A drug load of 1%, meaning that a particle of 100 mg contains 1 mg of active drug, is not uncommon. This could be problematic since a lot particles are needed which is expensive and could be harmful.

- The kinetics of the drug release are complicated and hard to predict since they are of non-zero order, affected by the drug and processing methods and even varies from patient to patient. See section 2.1.5 for more information. The initial burst release could be problematic as well as the drug might not be able to reach the target location.
- The adverse effect of nanoparticles on the human body is still vastly unknown and has given birth to a whole new field of science named nanotoxicology. More research is needed before the safety of nanoparticles can be guaranteed. See section 2.2.2 for more information.
- Nanoparticles need to be sterilized before applications and this affects the size and morphology which in turn changes the release profile. See section 2.2.3 for more information.
- A medical device does not only need to be effective to hit the market, it needs to be economically feasible as well. PLGA nanoparticles are relatively cheap to produce, but the research and tests required to obtain well defined properties might increase the costs. The scaling-up is also problematic since production steps like dialysis, ultracentrifugation and sonication are hard and even impossible to reproduce on an industrial scale.

2.2.2 Nanotoxicology

Nanotechnology is interesting because materials start to show different attributes and properties when the size is reduced to the nanoscale. The portion of surface atoms increases as well as the surface area, which often increases the surface energy and therefore also the material's ability to interact with surrounding matter, Cademartiri & Ozin (2009). From a biological prospective, the small size of nanoparticles allows them to interact with tissue and cells in a way that bigger particles are unable to do. Nanoparticles can pass through epithelium barriers, fine capillaries and are generally taken up efficiently by the cells, Lü et al. (2014). It is mainly these two attributes, the elevated reactivity and penetration, that make nanoparticles potentially harmful and as a consequence a relatively new subdiscipline of nanotechnology has emerged called *nanotoxicology*.

Not much is known about the toxicity of nanoparticles. Their interactions with biological systems are complex and often the *in vitro* results do not correlate well to the *in vivo* tests, Danhier et al. (2012). It is difficult if not impossible to provide an explanation on how nanoparticles in general affect humans. Focus should instead be aimed at understanding how each different type of nanoparticle acts in a biological system, Cademartiri & Ozin (2009). For example metallic particles will probably have different properties compared to ones made of polymers. One major concern about the safety of nanoparticles is difficulties in standardization. The synthesis process is often extremely sensitive since single atoms start to be significant at the

nanoscale, Cademartiri & Ozin (2009). McCall & Sirianni (2013) investigated how the size of PLGA nanoparticles changed between different synthesis occasions following the same protocol. A well-trained experimenter did seven batches and got a mean diameter of 340 nm±25 [1]. A second experimenter, trained by the first, followed the same protocol and got particles with a mean diameter of 328 nm±138. A third experimenter, who had worked with nanoparticles for a long time but independently of the others, synthesized particles with the same protocol and got mean size of 220 nm±70. The results show that the outcome of nanoparticle synthesis can differ even though the same protocol is being used.

The journal *Nature Nanotechnology* writes in an editorial about nanotoxicology that although the number of published papers in the field has increased by nearly 600% the last decade few of them offer any consistent results of value, Nanotechnology (2012). Comparison between studies is difficult due to lack of standards. The journal suggests that at least following parameters should be included in all papers to facilitate comparison: particle size and distribution, chemical composition, impurities, degree of nanomaterial aggregation or agglomeration during the experimental conditions, surface chemistry, surface area, morphology, surface reactivity and persistence.

To summarize, it is still mostly unclear how nanoparticles interact with the body. A lot of research is still required and each type of nanoparticles must be investigated individually. Nanotechnology is a growing field and nanotoxicology needs to keep up. Standardization of the toxicity tests seems to be the main challenge together with international collaboration in the work of regulating the field. Right now it is difficult to make any specific conclusions about the toxicity of nanoparticles, but a healthy approach is to consider a nanomaterial highly toxic until proven otherwise, Cademartiri & Ozin (2009).

2.2.3 Sterilization

All medical implants are required to be sterile before entering the body to reduce the risk of infections and other contaminations. A material is considered sterile if it has no more than a certain number of viable microorganisms that could pose a risk when administered. The current accepted sterility assurance level (SAL) for medical devices is limited to 10^{-6}, meaning that no more than one in a million units is allowed to have a single microorganism, Singh & Gulumian (2014).

A common sterilization technique for both laboratories and hospitals is autoclavtion where the materials are sterilized by heated steam (121 °C) during elevated pressure. This technique is ill-suited for PLGA since the polymer is heat sensitive with a low glass transition temperature (Tg), K.A.Athanasiou (1996). Tg is the temperature where the polymer chains start to move individually. When a polymer passes its Tg

[1] The results from the seven batches were 317 nm±99, 342 nm±112, 298 nm±104, 361 nm±110, 339 n±115, 360 nm±123, and 364 nm±110, which in my opinion should result in a mean diameter of 340 nm±110 and not 340 nm±25.

it goes from a glassy to a rubbery state and becomes soft and flexible. The steam also poses a problem since PLGA is hygroscopic and degrades through hydrolysis. The water penetrates the polymer and initiates the breakage of the bonds in the main chain in a reaction known as *chain scission*. A decrease of molecular weight therefore always follows autoclavation, K.A.Athanasiou (1996).

Chemical sterilization by ethylene oxide (EtO) gas is generally a good option for materials sensitive to heat and moisture, Singh & Gulumian (2014). EtO, see figure 2.4, denatures nucleic acids and functional proteins of most microorganisms including viruses. The gas is however toxic and carcinogenic for humans which might pose a threat for medical use. Residues of EtO could remain on the surface or inside the device in harmful quantities after sterilization. Adequate degassing and aeration are therefore required to make EtO sterilization applicable for medical devices, K.A.Athanasiou (1996). The risk of entrapment of EtO is present in PLGA devices due to the inherent porosity. EtO is therefore not a recommended sterilization method for PLGA devices intended for medical use.

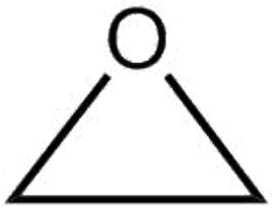


Figure 2.4: Ethylene oxide

Irradiation is another sterilization method which includes X-rays, UV-light, electron beam, γ-irradiation etc, where the latter is the most common form due its high penetration, Singh & Gulumian (2014). Irradiation is advantageous since it does not involve chemicals, heat or leaves any residues. γ-irradiation, sometimes written gamma irradiation, can effectively disinfect a device since it can penetrate through the device, although this ability might also change the properties of the material, Singh & Gulumian (2014). PLGA is sensitive to γ-irradiation and sterlization could cause instability, deterioration and breakage of the polymer chains, Lü et al. (2014). Spenlehauer et al. (1989) investigated the effect of γ-irradiation on PLGA microparticles and their results showed that it caused a 30-40% decrease in molecular weight, a burst drug release after 8 days compared to 60 days for non-irradiated samples and might reduce storage capability. The study tested radiation doses of 28.4 and 37.7 kGy (1 Gray = 100 rad = 1 joule of radiation energy per kilogram of matter) but no significant difference could be observed. Even though γ-irradiation involves some complications it remains the most common method for terminal sterilization for PLGA nanoparticles, Lü et al. (2014).

Since no sterilization technique right now is optimal a lot of studies are still focused on developing suitable sterilization methods for PLGA devices. Filtration is a promising candidate since it does not involve any heat and water or leaves residues. Successful attempts have been made with 0.22 μm filters, although low yields could

occur since PLGA nanoparticles might clog and form aggregates, Singh & Gulumian (2014). This is circumvented by careful control of the particle size and size dispersity, a feat that is not always possible. Other sterilization methods that have been investigated are ethanol, peracetic acid and antibiotic solution where none have shown to be ideal, Lü et al. (2014).

2.2.4 Nanoparticles synthesis

A lot of research has been conducted on improving existing and finding new methods for producing nanoparticles. The aim of these studies could be to:

I. Control the properties of the particle, e.g. size, morphology and drug release behavior.
II. Improve the synthesis, e.g. cheaper, more environmentally friendly, increased monodispersity.
III. Enhance drug efficiency, e.i. make sure that the drug remain active and stable even after the synthesis.

Three main methods are used for synthesizing PLGA nanoparticles intended for drug encapsulating; emulsion techniques, phase separation and spray drying, Mundargi et al. (2008). They all have their advantages and disadvantages and therefore many more promising techniques are being researched and developed. Here follows a summary of the current synthesis methods.

Solvent evaporation method
The single emulsion method is the most common technique to synthesize PLGA nanoparticles, Sharma et al. (2011). The process involves one organic and one aqueous phase and is often denoted oil-in-water or simply O/W. PLGA is initially dissolved in a volatile organic solvent, e.g. dischloromethane, chloroform or ethyl acetate, H.K. Makadia (2011), Sharma et al. (2011). The drug is then added to the solution to form a dispersion. This organic phase, now containing polymer and drug, is added to a large volume of aqueous phase. The water now acts as a continuous phase that suspends the droplets of the organic dispersion which is called the dispersed phase. The two phases form an emulsion with the aid of stirring, vortexing and/or sonication, McCall & Sirianni (2013). The aqueous phase often contains an emulsifier to stabilize the emulsion. Polyvinyl alcohol (PVA) is the most common but other surfactants like polysorbate 80, poloxamer 188 and vitamin E TPGS (d-α-tocopherol polyethylene glycol succinate) are used as well. The organic phase is then allowed to evaporate by increased temperature, under pressure or continuous stirring, Alimohammadi & Joo (2014), and the nanoparticles can then be collected by centrifugation, McCall & Sirianni (2013).

The single emulsion method is most suitable for hydrophobic drugs like steroids since the drug is dispersed directly in the polymer solution. If the drug is instead hydrophilic, for example peptides, proteins and vaccines, a technique called the double emulsion method can be used instead, H.K. Makadia (2011). The method adds a step to the single emulsion method to form a water-in-oil-in-water (W/O/W) emulsion. The drug is first dissolved in an aqueous phase and then added to an

organic phase containing dissolved PLGA. Mechanical stress is applied and a primary water-in-oil emulsion is formed. This W/O emulsion is the objected to the same steps as the organic phase in the single emulsion technique, McCall & Sirianni (2013), H.K. Makadia (2011), Alimohammadi & Joo (2014), Sharma et al. (2011).

The main advantage of the solvent evaporation methods is that they are fairly straight forward and can be performed in most labs. The techniques also come with some complications. The nanoparticles synthesized are susceptible to aggregation, oxidation and cleavage, especially in the interface between the organic and the aqueous phase, Mundargi et al. (2008). The emulsifiers used might be difficult to wash away and could have a negative impact on particle degradation and its interaction with cells, Mundargi et al. (2008).

Phase separation (Coacervation)
Coacervation is a process in which two liquids are phase separated. In PLGA nanoparticles synthesis the coacervate phase contains the particles and the supernatant phase is depleted from polymer, H.K. Makadia (2011). Just like in the solvent evaporation method an O/W emulsion if formed for hydrophobic drugs and W/O/W for hyrdophilic ones. Organic medium is then gradually added while stirring which extracts the polymer solvent. The result is a phase separation of polymer and formation of a soft so called coacervate of drug containing droplets. The droplets are quenched by dipping the coacervate quickly in an insoluble medium, Sharma et al. (2011). A drawback with this technique is that the particles tend to agglomerate. Another problem is the large amount of organic solvent required which needs to be removed from the particles.

Spray drying
In spray drying, the nanoparticles are formed by spraying a W/O emulsion in a hot stream of air, Sharma et al. (2011). The method has many advantages. It is rapid, includes few process parameters and been showed to be able to encapsulate many different types of drugs without significant loss in their biological activity, H.K. Makadia (2011). These attributes makes spray drying stand out as one of the few PLGA nanoparticle synthesis methods that could be used on an industrial scale. The main disadvantage is the adhesion of newly formed nanoparticles on the inner wall of the spray-dryer which hinders effective particle collection, Sharma et al. (2011).

Emulsification solvent diffusion
In the emulsification solvent method the organic and aqueous phase are both mutually saturated at room temperature before use to ensure the initial thermodynamic equilibrium of both liquids. An O/W emulsion is then formed with the aid of a high-speed homogenizer. Water is subsequently added under regular stirring resulting in phase transformation and outward diffusion of the solvent from the internal phase, leading to the nanoprecipitation of the polymer and the formation of colloidal nanoparticles. The emulsification solvent diffusion method offers several advantages, for example high encapsulation efficiencies, high batch-to-batch reproducibility and

narrow size distribution. However two drawbacks are the big quantities of water required and leakage of hydrophilic drugs into the aqueous phase, Alimohammadi & Joo (2014).

Nanoprecipitation
Nanoprecipitation, also known as the solvent displacement method, involves three components; the polymer, the polymer solvent, and the nonsolvent of the polymer. Polymers and drugs are dissolved in a polar, water-miscible solvent, for example acetone, ethanol, or methanol. The solution is added dropwise into an aqueous solution with surfactant. The solvent diffuse away rapidly which causes immediate formation of nanoparticles. Because of the rapid formation of particles without any need of homogenization nanoprecipitation is called a one-step process.

Several other methods of synthesizing PLGA nanoparticles exist as well. Salting-out processes can be utilized and dialysis can be used to prepare nanoparticles with narrow size distribution. A new and interesting approach is to work with supercritical fluids. The phenomena of supercritical fluids occurs when a substance is at a pressure and temperature above its critical point. The substance is then neither gas nor fluid but can display properties of both states. Supercritical fluid technology can be used to produce polymeric nanoparticles and has been showed to be more environmentally friendly and produce particles with high purity, Sharma et al. (2011).

2.2.4.1 Synthesis parameters

Even though extensive research has been conducted on PLGA nanoparticle synthesis the understanding of the process parameters is still lacking, Sharma et al. (2011). The results are sometimes difficult to interpret or even contradictory. Here follows some of the process parameters and a summary of their impact on the particle synthesis.

PLGA molecular weight
The initial molecular weight of the polymer seems to have a small effect on particle size. A study showed that 50:50 (LA:GA) PLGA nanospheres went from 102 ± 4nm to 154 ± 17 nm when the molecular weight increased from 12 kDa to 48 kDa, Dinarvand et al. (2011). However another study using 50:50 PLGA with a molecular weight of 12, 53 and 143 kDa achieved the mean sizes of 563 ± 6, 685 ± 40 and 375±22 respectively, Prabha & Labhasetwar (2004). The results strongly suggests that molecular wight has an impact on particle size, but the nature and extent is still unclear.

PLGA concentration
According to Wang et al. (2012) an increased concentration of PLGA in the organic phase results in bigger particles. Four different PLGA concentration were tested and the particle size increased linearly from 257 nm (2% w/v) to 567 nm (8% w/v). Enhanced viscosity is a consequence of higher concentrations, and it leads to decreased dispersion which could explain why the particles got bigger. Drug loading

and encapsulation efficiency were better at lower concentrations.

Organic solvent
Dichloromethane and ethylene acetate are two common solvents for PLGA in nanoparticle synthesis and exhibit different properties. Ethylene acetate is more hydrophilic and is partly-miscible in water (10% (v/v) at 25°C) while dichloromethane is almost immiscible (2% (v/v) at 25°C), Song et al. (2006). It is still possible to dissolve PLGA in ethylene acetate, however it is easier to do in dichloromethane. The interfacial tension between the solvent and water is 28.3 mN/m for dichloromethane and 1.7 mN/m for ethyl acetate, Vineeth et al. (2014), meaning less force is required to form new surface which facilitates emulsification. Studies by Vineeth et al. (2014) and Song et al. (2006) showed that ethyl acetate produces smaller particles in comparison with dichloromethane and held lower interfacial tension and viscosity as reasons. This behavior was more pronounced when an ionic stabilizer (didodecyl dimethyl ammonium bromide, DMAB) was used compared to a non-ionic stabilizer (polyvinyl chloride, PVA), implying that the miscibility of the solvent is more significant for particle size when the particles are stabilized by electrostatic repulsion rather than steric hindrance, Song et al. (2006). Ethylene acetate could even form a stable emulsion and thus produce nanoparticles without a stabilizer, Vineeth et al. (2014). However, the low volatility of ethylene acetate affects the solvent removal and yield negatively, Sah & Sah (2015).

It is not only a matter of achieving the desired results, how we get there is just as important. This way of sustainable thinking is sometimes called *green chemistry*. The aim of green chemistry is to reduce or eliminate generation of hazardous substances in chemistry. For example, dichloromethane is an ozone-depleting halogenated solvent which is carcinogenic, Sah & Sah (2015). Ethyl acetate is a much less harmful solvent and is therefore considered a better option.

Stabilizer
A stabilizer, or emulsifier, is often added during the particle synthesis to obtain a stable emulsion. These are often surfactants which will be orientated towards the interface between the organic and aqueous phase due to their amphiphilic nature. The type of stabilizer affects the result. Song et al. (2006) showed that an ionic stabilizer, didodecyl dimethyl ammonium bromide (DMAB), produced small PLGA nanoparticles when a partially water-soluble organic solvent like ethylene acetate was used. The particle size increases on the other hand drastically with a fully water-soluble solvent like acetone or a fully water-immiscible solvent like dichloromethane. These changes were not apparent when non-ionic stabilizers like Pluronic F68 and polyvinyl chloride (PVA) were used. These stabilizers produced particles of medium size irrespective of solvent used.

PVA is the most commonly used stabilizer for PLGA nanoparticle synthesis, but the surfactant has some disadvantages. PVA forms an interconnected network with the surface of the PLGA particles which makes the surfactant next to impossible to remove even with extensive washing. It has been showed that particles with high

amounts of residual PVA had lower cellular uptake, possibly due to their more hydrophilic surface. The same study also showed that the ζ-potential (see section 2.4.3 for more information about ζ-potential) decreased with increased PVA concentration due to shielding of the negative carboxyl groups of PLGA, Sahoo & Labhasetwar (2002). McCall & Sirianni (2013) recommends the use of vitamin E- D-α-Tocopherol polyethylene glycol succinate (TPGS) over PVA since emulsification and drug encapsulation are improved.

Stabilizer concentration
Polyvinyl chloride (PVA) is as mentioned the most common stabilizer in PLGA nanoparticle synthesis. The amount of PVA used has a high impact on the end results. Higher concentrations decrease particle size and might improve monodispersity, but also cause more PVA residues on the surface of the particle which impairs cellular uptake, Prabha & Labhasetwar (2004). Sahoo & Labhasetwar (2002) showed that when the PVA concentration was varied from 0.5% (w/v) to 5% (w/v) the mean size of the nanoparticles varied from 522 nm to 380 nm . One explanation for this is that smaller spheres have a bigger surface area compared to larger with the same total volume, therefore more surfactants are required to achieve small spheres. Similar results can has been showed for poloxamer 188 by P. Yan (2002).

Sonication
A sonicatior is often utilized to transfer energy in the form of sound waves to a mixture in order to form an emulsion. Higher sonication intensity results in a finer emulsion which in turn produces smaller particles, McCall & Sirianni (2013). Not only intensity but also sonication time has an impact on the results. Wang et al. (2012) showed that the particle size decreases with sonification time up to 40 sec and drug loading improved up to 60 sec, after which no significant further improvement could be observed.

Inner aqueous volume
Wang et al. (2012) measured the effect of particle size and drug loading with varying the volume of the water phase from 0.1 ml-0.4 ml. The size varied from 250-385 nm while the drug loading capacity increased with bigger water phase.

2.2.5 Osteogenesis

Osteogenesis, or ossification, is the process of bone formation. Extensive research has been conducted on PLGA biomaterials for bone healing, especially in the form of scaffolds. Scaffolds are synthetic bone substitutes and have been showed to be a promising alternative to autografts (tissue from the patient), allografts (tissue from a donor) and xenografts (tissue from a different species), Gentile et al. (2014). Metallic scaffolds possess suitable mechanical properties for load-bearing applications and ceramics exhibit excellent biocompatibility, but neither is generally degradable, Gentile et al. (2014). They also risk to weaken surrounding healthy bones and hamper the formation of new bone due to a phenomena called *stress shielding*, Morrison

et al. (1995). Just like muscles, bones need stimuli to grow. If the implant carries all the load normally carried by the bone there is no need for the body to waste resources forming new bone tissue or retaining existing one, J.S. Temenoff (2008). PLGA scaffolds on the other hand degrade with time which gradually decreases the mechanical properties and thus transfers more and more stress from the implant to the surrounding bone tissue. Another advantage of degradable implants is that they do not need to be surgically removed after healing. Even implants that are meant to be permanent might require surgery due to uncontrolled degradation.

However, PLGA scaffolds display some disadvantages as well. Pure PLGA has not the mechanical properties to be used in load bearing applications and also possesses poor osteoconductivity (bone growth on a surface), Gentile et al. (2014). PLGA is therefore used in combination with other materials. One such example is a composite of PLGA and nanohydroxyapatite which has showed to promote osteoinduction (bone formation) on human mesenchymal stem cells (hMSC:s), Lock & Liu (2011). The bioactivity of pure PLGA scaffolds has received little attention according to Di Toro et al. (2004) who investigated how human osteoblasts were affected by growth on a PLGA surface. Their conclusion was that human the PLGA had no negative (nor positive) effect on cell morphology, viability and proliferation.

It seems that PLGA scaffolds might not induce osteogenesis, but the same is not necessarily true for PLGA nanoparticles. An article by Jiang et al. (2015) investigated how PLGA nanoparticles affected the differentiation of rat MSCs. This is a fairly new field according to the authors who state: "*However, the influence of PLGA particles on cell phonotype and/or differentiation has not been addressed so far.*" (2015). Their conclusion was that PLGA nanoparticles indeed could induce osteogenic differentiation of rat MSCs. This promising result and the novelty of the topic encourage further research on the effect of PLGA nanoparticles on the differentiation of *human* mesenchymal stem cells, which is the subject of this thesis.

2.3 Human mesenchymal stem cells

Human mesenchymal stem cells, henceforth referred to as hMSCs, are multipotent stem cells. They can be isolated from the bone marrow and propagated in culture and have the ability to differentiate into almost any connective tissue, namely adipocytes (fat cells), chondrocytes (cartilage cells) and osteoblasts (bone cells), Alberts (2008). The differentiation can be controlled and hMSCs have therefore attracted attention in tissue engineering, Jiang et al. (2015).

Osteogenesis is the focus of this study and osteoblasts are therefore the cell type of interest. Osteoblasts form new bones by first secreting an uncalcified bone matrix called *osteoid*. The osteoid is rich in type I collagen, a tough fiber which resists pulling forces. The osteoblasts also secret calcium phosphate in the form of hydroxylapatite crystals, which resists compression. The osteoid is rapidly calcified and converted to hard bone, a durable composite material, Alberts (2008). hMSCs can be differentiated into osteoblasts by adding certain substances to the

cell medium, the most common ones being dexamethasone, ascorbic acid and β-glycerophosphate. Dexamethasone induces Runx2, Langenbach & Handschel (2013), a gene which promotes osteogenesis, Alberts (2008). Ascorbic acid, more commonly known as vitamin C, enhances secretion of type I collagen while β-glycerophosphate is the source of phosphate in hydroxylapatite, Langenbach & Handschel (2013).

2.4 Experimental methods

Several different analyzation tools and techniques were used this work to verify successful nanoparticle formation and cellular differentiation and to obtain information about the particles and the cells such as size, morphology and viability. This chapter contains brief summaries of these techniques in order to facilitate interpreation of the results presented in chapter 4.

2.4.1 Scanning electron microscopy

Scanning electron microscopy (SEM) utilizes electrons to get a visible representation of the sample. An electron beam scans the sample and the interaction causes other signals, for example secondary electrons, auger electrons and X-rays, to be emitted from the surface. These signals can then be analyzed to obtain information about surface topography and composition, Nanoscience Instruments (2016). The main advantage of electron microscopes is that they, as the name suggests, use electrons. The maximum resolution is, among other factors such as the quality of the lens, depended on the wavelength. Optical microscopes are limited by the wavelength of visible light and have a theoretical limit of resolution of about 200 nm. Electrons have much shorter wavelengths and SEM can have a resolution down to 1 nm, Nanoscience Instruments (2016). One drawback with using electrons is that the sample need to be conductive. Non-conductive samples can be made conductive by sputter coating with a thin layer (typically 2-20 nm) of a metal, commonly gold, palladium or platinum.

2.4.2 Dynamic light scattering

Dynamic light scattering, abbreviated DLS, is a technique to measure size and size distribution of colloids in a suspension. The technique measures how monochromatic light scatters from the particles and how the intensity of the light fluctuates over time. The rate of fluctuation correlates to the speed of the Brownian motion of the particle through the suspension. Brownian motion is caused by random collision of the solvent molecules on the particles. Smaller particles are more affected by this bombardment and travel therefore faster which causes the intensity of the scattered light to fluctuate more rapidly. From this data it is possible to use correlation functions to calculate the translational diffusion coefficient D. The hydrodynamic diameter, $d(H)$ can then be obtained by using D in the Stokes-Einstein equation, Malvern Instruments (2012), see Equation 2.1

$$d(H) = \frac{kT}{3\pi\eta D} \tag{2.1}$$

where *d(H)* is the hydrodynamic diameter (m), k is Boltzmann's constant ($\frac{J}{K}$), η is the viscosity ($\frac{kg}{ms}$) and D being the translational diffusion coefficient ($\frac{m^2}{s}$).

The hydrodynamic diameter is not the actual diameter of the particles, but the diameter of a hypothetical hard sphere which diffuses at the same speed as measured. Therefore it is crucial to validate that the particles are indeed spherical before DLS measurements. For a non-spherical particle, DLS will give the diameter of a sphere that has the same average translational diffusion coefficient as the particle being measured. The ionic concentration of the medium affects the results as well. Ions affect the thickness of the electric double layer, i.e the Debye length, which is the distance at which the electrostatic effects of the particle cease to affect the medium. Low concentrations of ions will not efficiently shield the charge of particle and thus produce an extended double layer which reduces the diffusion speed, resulting in a larger apparent hydrodynamic diameter. It is therefore advised to have an ionic concentration of at least 10mM to reduce the Debye length in order to increase the accuracy of the measurements, Shaw, R. Malvern Instruments (2014).

2.4.3 ζ-potential

ζ-potential, sometimes written zeta potential, is a way to describe the electric charge of a particle. All materials spontaneously obtain a surface electric charge in a polar medium like water. ζ-potential is not the same as surface charge. Instead it is the charge of the slipping plane, also known as the shear plane, see figure 2.5. The medium beneath the slipping plane is attached to the particle and moves with it. One could argue that ζ-potential is a more interesting property than surface charge since it is the charge which other molecules and particles "see" and interact with, Particle Sciences (2012).

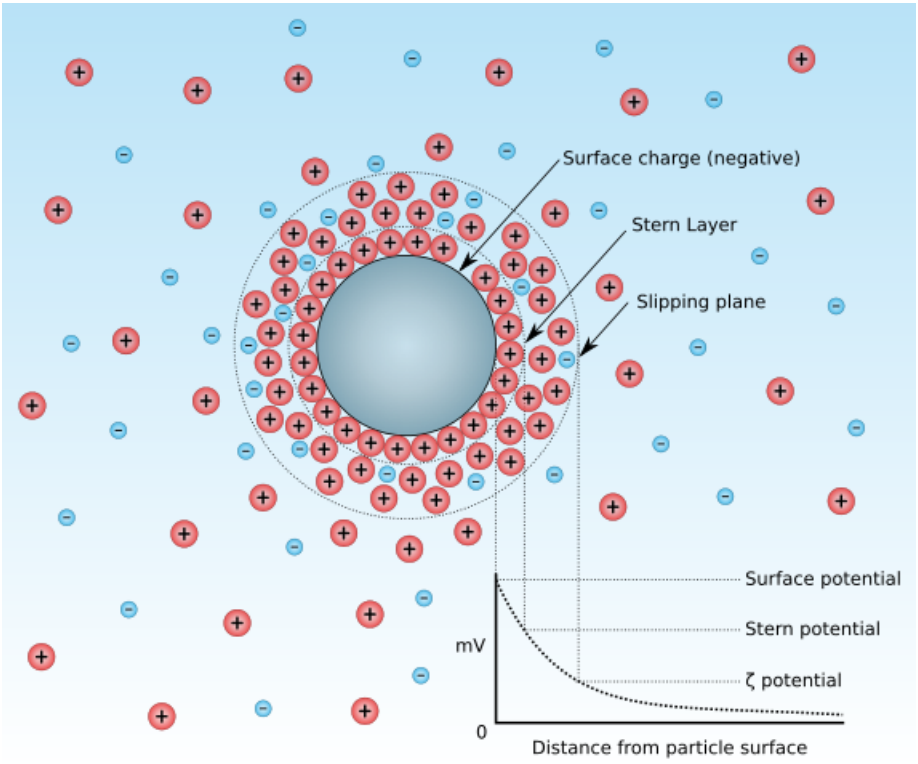


Figure 2.5: Electrostatic interactions of a negatively charged particle in a polar medium. The stern layer contains only cations. The slipping plains conatins both anions and cations which move with the particle.
Source:
http://en.wikipedia.org/wiki/File:Zeta_Potential_for_a_particle_in_dispersion_medium.png

The value of the ζ-potential gives an indication of the stability of the suspension. Equal charges repel each other and the repulsion forces of particles with high ζ-potentials are more likely to overcome the existing attraction forces such as van der Waals or hydrophobic forces. Such particles will therefore not aggregate and the suspension is electrostatic stabilized, Particle Sciences (2012).

2.4.4 CCK-8

CCK is an abbreviation for Cell Counting Kit. The '8' refers to the water-soluble tetrazolium salt WST-8 utilized. WST-8 can be reduced by an electron mediator and will then form an orange water-soluble formazan dye. The amount of formazan dye produced is directly proportional to the number of living cells because of following redox-chain: dehydrogenases (hydrogen-removing enzymes) inside the cells transfer hydrogen to electron acceptors (usually NAD) which in turn reduces extracellular electron mediators who in the end can reduce WST-8. The amount of living cells can then be estimated by measuring the absorbance with a spectrophotometer, Dojindo Molecular Technologies (2016).

2.4.5 ALP-staining

Alkaline phosphatase, often abbreviated ALP, is as the name suggests an enzyme which catalyzes the removal of phosphate groups, Alberts (2008), in an alkaline environment. The dephosphorylation is achieved through hydrolysis and involves magnesium and zink, Millán (2006). Alkaline phosphatase is present in many different tissue types, but the expressed levels are increased in the early stages of osteogenic differentiation, He et al. (2016). Human mesenchymal stem cells can be stained with a BCIP/NBT, a substrate which colors the cells blue-violet in the presence of alkaline phosphatase. ALP-staining is therefore a suitable method to detect initial osteogenic differentiation. The test can also be made quantitative by measuring the absorbance with a spectrophotometer.

2.4.6 Alizarin Red S

Osteblasts deposit calcium in the extra cellular matrix during osteogenesis. Calcium is the main inorganic component in bone and provides mechanical strength in form of rigidity and hardness. Calcium is also stored in the bones and can be accessed by the body where it has many roles, for example as an intracellular signal (i.e. second messenger) and as a cofactor in the blood coagulation cascade. Calcification is therefore an indication of osteogenesis and calcium in the extra cellular matrix can be stained with Alizarin Red S (ARS), where the 'S' indicates that a sulfonate group has been attached to the molecule as depicted in figure 2.6. The calcium ion forms a complex with ARS in a chelation process (a chelate is a complex with a central metal atom surrounded by ligands, Atkins & Jones (2009)). The result is a red-orange stain where extracellular calcium is present. The staining is non-specific, but other metal ions such as magnesium and iron do not normally occur in sufficient concentrations to have a significant impact on the result, IHC World (2016). The staining can be both qualitative and quantitative.

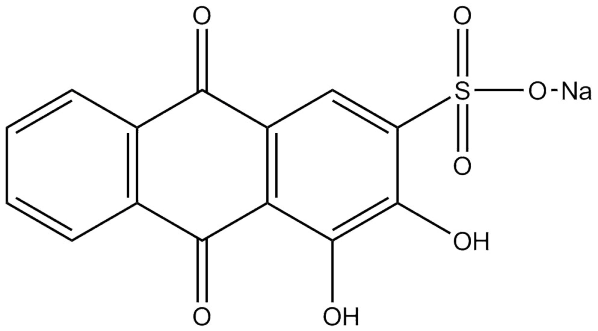


Figure 2.6: Molecular structure of Alizarin Red S

3
Method

The main goals for this study have been to synthesize nanoparticles of PLGA and investigate how different concentrations of said particles affect the osteogenic differentiation of human mesenchymal stem cells. The method is divided into two major parts; nanoparticle synthesis and *in vitro* tests. In the first part the synthesis of PLGA nanoparticles was investigated and optimized. The result was spherical PLGA nanoparticles, approximately 400 nm in diameter and with a narrow size distribution. These particles were then used in the second part. Human mesenchymal stem cells (hMSCs) were exposed to the particles in different concentrations to examine the viability and the effect on osteogenic differentiation. The stem cells were differentiated into osteoblasts and the osteogenic activity was measured. The aim was to investigate if the PLGA nanoparticles could induce and/or enhance osteogenesis of human mesenchymal stem cells. The outcome of the tests could be relevant both in tissue engineering and drug delivery.

Section 3.1 depicts the method used to obtain the results presented in chapter 4. A summary of the project work can be read in section 3.3. This section depicts a summary of all the tests that were performed during the project together with thoughts and ideas. The aim of this section is to give the reader an understanding of how the project progressed; what have been done, why it was done and how it went. It also functions as a testimony of why the final method was chosen and why the aim of the project was changed. A more extensive summary of the progress of the project is found in appendix B.1.

3.1 Particle synthesis

This section depicts the synthesis of the PLGA nanoparticles. The protocol was refined during the project and the one presented here is the final version. See section 3.3 and appendix B.1 for an extensive insight of the optimization process.

3.1.1 Synthesis protocol

Poly(lactic-co-glycolic acid), henceforth referred to as PLGA, nanoparticles were synthesized by the single emulsion method. PLGA with a molecular weight of 70-88 kDa was dissolved in ethyl acetate to obtain a polymer concentration of 100 mg/ml. PLGA with a molecular weight of 20 kDa and dichloromethane as organic solvent

were investigated as well but not used in the *in vitro* tests. 2 ml of the solution was added dropwise to 4 ml of an aqueous phase with 1% polyvinyl alcohol (molecular weight: 30-70 kDa, degree of hydrolysis: 87-90%) while vortexing. Other concentrations of polyvinyl chloride as well as another stabilizer, E TPGS (d-α-tocopherol polyethylene glycol succinate), were tested during the optimization. The sample was quickly transferred to a sonicator and sonicated on ice at 50 W for 3·10 s with 10 s rest between each interval. Other sonication settings were used during the optimization phase. The newly formed emulsion was added to 100 ml diionized water containing 0.1 % polyvinyl alcohol to facilitate evaporation of ethyl acetate. The solution was stirred in an open beaker for 24 h. After the evaporation step the particles were collected through a series of washing steps. The sample was centrifuged at 12 kRPM, which equals 7.2 kG, for 10 min at 20 °C. The supernatant was discarded and the pellet resuspended in diionized water using vortexer and water bath sonication. The washing step was repeated three times in order to remove excess polyvinyl alcohol. An aliquot of the sample was separated for future SEM imaging. Trehalose, a cryoprotectant, was added to the remaining sample in ratio of 2:1 polymer:trehalose. The sample was frozen at 80 °C for 3 h. Afterwards the sample was quickly transferred to a lyophilizer and freeze-dried at -42 °C and 9.8 Pa for 48 h. Lab tissue was used as a filter to allow sublimation of water while retaining particles during the lyophilization. The particles were later sterilized by γ-irradiation (20 kGy) and stored in freezer at 80 °C.

3.1.2 Particle characterization

For SEM imaging, a thin layer of dry sample was sputter-coated with platinum (5 min) and studied using a voltage of 5 kV. Gold-sputtering was also investigated but yielded no results. DLS measurements of the particles were carried out at 25 °C in three different mediums; deionized water with 10 mM KCl as an electrolyte, cell medium (DMEM LG) and cell medium with 10% filtered serum (FBS). The sample was added to the medium until it became slightly turbid before measurements began. The ζ-potential was measured at 25 °C in deionized water with particle concentrations enough to cause just slight turbidity.

3.2 *In vitro* tests

hMSCs of passage 5 were thawed and expanded for the *in vitro* tests. The medium was changed every second day and consisted of basal medium with 10% filtered fetal bovine serum (FBS), 1% antibiotics (penicillin and streptomycin) and 1% L-glutamine. The cells were expanded until reaching passage 7 and 10 whereupon they were exposed to PLGA nanoparticles with a diameter of approximately 400 nm. The nanoparticles were introduced via a testing medium in the following concentrations: 10, 50, 100, 250 and 500 µg/ml. The testing medium consisted of DMEM LG with 10% filtered FBS and 1% antibiotics (penicillin and streptomycin). The cells were incubated with the particles for 24 h after which the medium was changed to a

testing medium free of PLGA nanoparticles.

3.2.1 Cytotoxicity

The cytotoxicity of the PLGA nanoparticles was investigated using a CCK-8 test. In short, non-differentiated hMSCs of passage 10 in a 24 well plate were washed two times with phosphate buffered saline (PBS) before addition of testing medium with 10% CCK-8 solution. The cells were incubated in dark conditions for 4 h after which the OD value was measured with a spectrophotometer at a wavelength of 450 nm. Cell viability was investigated after 1, 3, and 5 days and each measure point had 4 repeats.

3.2.2 Cellular differentiation

Osteogenic differentiation was induced on half of the remaining hMSCs by switching to a differentiation medium, consisting of testing medium with the addition of 1% β-glycerol phosphate disodium salt hydrate (10 mM), 0.1% ascorbic acid (50 mg/ml) and 0.1% dexamethasone (0.1 μM). Testing medium without osteogenic promotors was used on the remaining cells as a control.

The enzyme alkaline phosphatase was stained using an ALP-staining kit 14 days after osteogenic differentiation had been induced. In short, both differentiated and non-differentiated hMSCs of passage 7 in a 48 well plate were washed two times with PBS. The cells were fixated by adding 200 μl paraformaldehyde (4% w/v in PBS) to each well. After 30 min the cells were washed two times with PBS. 200 μl of ALP-staining solution was then added to each well. The plate was covered in metal foil and kept in dark for 30 min after which the cells were washed two times with PBS and studied in a light microscope.

Extracellular calcium deposits were stained using an Alizarin Red S staining-kit 21 days after osteogenic differentiation had been induced. In short, both differentiated and non-differentiated hMSCs of passage 7 in a 48 well plate were washed, fixated and washed again in the same manner as for the ALP-staining. 200 μl Alizarin Red S staining solution (0.1%, pH 8.3) was added to each well. The plate was then covered in metal foil and incubated at room temperature for 10 min with gentle shaking. The dye was then carefully removed using a pipette. The wells were washed extensively with deionized water until the dye ceased to stain the washing water. The cells were then analyzed qualitative in a light microscope. The dye was later desorbed for quantitative studies. 300 μl hexadecetylpyridinium chloride monohydrate (10% w/v in 10 mM in sodium phosphate buffer, pH 7.0) was added to each well and incubated for 1 h with gentle shaking. 100 μl of each well was then transferred to a 96 well plate and the OD was measured at a wavelength of 562 nm using a spectrophotometer.

3.3 Project summary

The very first weeks of the projects were designated to be familiar with the lab and the synthesis protocol. A literature research was conducted immediately to obtain knowledge of how the synthesis parameters affect the outcome, but particles synthesis was carried out simultaneously to gain practical experience.

Since both theoretical knowledge and practical experience were lacking in the beginning, the major purpose of the first synthesis was to get a feeling for the parameters and evolve the laboratory skill. The first synthesis produced clearly visible particles in the centimeter scale. Electron microscope images of the second synthesis showed however spherical particles close to the nanoscale. The particles were still too big and polydispersive (large size distribution), but it was a big improvement from the first synthesis. The mood was optimistic because of the promising result after only two tries and it was expected that just some further optimization was required.

A lot of experiments were performed the following weeks to understand how the parameters affected the outcome in order to gain control of the synthesis. A test matrix was established and one parameter was changed at a time to deduce its effect. Unfortunately very little information could be extracted from these tests since the scanning electron microscopy (SEM) images showed no particles. Instead they displayed a sponge-like network with traces of spheres. It was assumed that the particles had melted together.

This delayed the project plan since no results could be produced until the problem with the melting particles had been solved. Another time-consuming factor was the availability of the electron microscope. SEM-time was only available every second week resulting in long waiting times before the effects of changes in the protocol could be observed. Dynamic light scattering (DLS) is another method to characterize the the particles, which is fast, cheap and available. However, the method only gives reliable results if the particles are spherical. For this reason the plan was to use it when the morphology of the particles had been established through SEM. But after weeks of no usable SEM images DLS was tried out anyways and the quality of the results suggested that the particles were indeed spherical.

The following conclusion was that the fault was not in the synthesis but in the sample preparation for the SEM. Different approaches were tested, for example if the sample should be dry or dispersed, but to no avail. The SEM images still continue to show a network of melted particles. This was starting to become a major problem due to the time limit of the project. It was also somewhat mysterious why the good particles synthesized in the beginning of the project could not be reproduced.

The solution came from another research group. A colleague was also working with PLGA nanoparticles and he suggested to use platinum instead of gold for coating for SEM. Apparently the gold sputtering process evolved too much heat which caused the PLGA nanoparticles to melt. It was later confirmed that the good images ob-

tained in the beginning of the project had also been sputtered with platinum instead of gold. Why this had not surfaced earlier was because the imaging was made by another researcher since I had not undergone SEM training yet.

Platinum was henceforth used and new SEM images were made of both old and new samples. The results were generally good and showed spherical particles in the nanoscale with relatively low size distributions. Now when it was confirmed that the particles were indeed spherical DLS was used for quick characterization because it is faster, cheaper and more available than SEM. The next challenge was to control the size of the particles. It was desired to synthesize at least two different sizes in order to investigate if the cell interaction was size-dependent. Somewhat surprisingly, it proved more difficult to produce bigger particles. Small particles ranging from 100-600 nm were achieved immediately but the size could not be increased further with the chosen synthesis method.

Another problem that arose was insufficient yield of the smaller particles. DLS and SEM measurements proved that particles of sizes of 150 nm and 450 nm could be produced with no overlap in size distribution, a requirement for the sizes to be deemed significantly different. However the yield of the smaller particles was too low to provide enough particles for both characterization and cell tests. The batch-to-batch variation was not insignificant and therefore it was desired to use particles from the same batch for all the tests. Due to this and lack of time it was decided to only test particles of one size. Size dependence is still important to investigate and should be studied in future work.

Now when suitable particles could be produced it was high time to start with the cell tests. The particle synthesis had already consumed a majority of the project time and even when several of the planned cell tests were discarded the remaining time proved to be insufficient. It was therefore decided to prolong the stay in China and at Tsinghua University with one month in order to obtain at least some results from the cell tests.

The cell tests begun with a training period where MC3T3 cells were used. MC3T3 is an osteoblastic cell line derived from mice and is a good substitute to human mesenchymal stem cells (hMSCs) since it can also differentiate into osteoblasts and is significantly cheaper. The cells were successfully thawed but growth was slow. The training period was very good to get to know the cell lab, the routines and how to avoid infections. After a cell viability test (CCK-8) on the MC3T3 cells I felt prepared to start to work with the hMSCs.

The cells were successfully thawed and expanded without any infections. PLGA nanoparticles were introduced in different concentrations to the cells and osteogenic differentiation was induced. The viability tests did not display any harmful effects of neither the particles nor the concentrations, although the variation was very large. The osteogenic differentiation was investigated by Alizarin Red S staining and ALP staining. Alizarin Red S stains the calcium in the extra cellular matrix (ECM) and

ALP is an abbreviation for alkaline phosphatase which is a protein whose expression is elevated in the early stage of osteogenic differentiation. The Alizarin Red S staining displayed a clear difference between non-differentiated cells and differentiated, thus suggesting the differentiation was successful. A clear difference could also be seen between the concentrations. Just by visual inspection it was clear that the staining intensity was increased with particle concentration. This is a very interesting result, suggesting that osteogenesis could be enhanced by high concentration of PLGA nanoparticles. However when the intensity of the staining was quantified using a spectrophotometer the complete opposite relationship was observed, calcium deposits decreased with concentration. Remaining nanoparticles could be an explanation of these contradictive results.

The ALP staining was successful but showed no difference between differentiated and non-differentiated samples nor between the different concentrations. All cells expressed alkaline phosphatase and the difference in amount was too small to be observed in microscope. Unfortunately no quantitative measurements were performed.

To summarize, the nanoparticles synthesis was successful but can be optimized further. The time available for cell tests was too limited to obtain satisfactory results. The few results that were extracted were hard to interpret due to high variation or contradictions. It seems that PLGA nanoparticles are relatively harmless for hMSCs and do affect the osteogenic differentiation. It is clear that more research is required and that there is a lot of potential for further discoveries in this field.

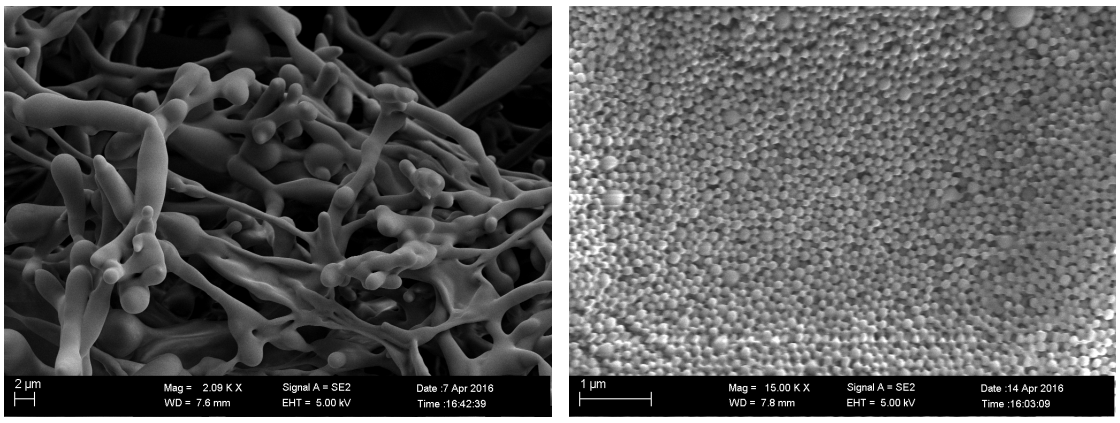
4
Results

This section consists of the relevant results from the PLGA nanoparticle synthesis and its optimization, together with results from the experiments where the effect of PLGA nanoparticles on the osteogenic differentiation of hMSCs was investigated. This project generated considerable amounts of other results as well, for example all the different batches of nanoparticles were characterized and several tests were performed during training with MC3T3-cells. Some of these results are found in appendix B.1. If not they have been deemed insignificant.

4.1 Synthesis optimization

The synthesis procedure was optimized during the project. The protocol was changed continuously and the parameters were investigated. The results from this optimization phase are presented in this section.

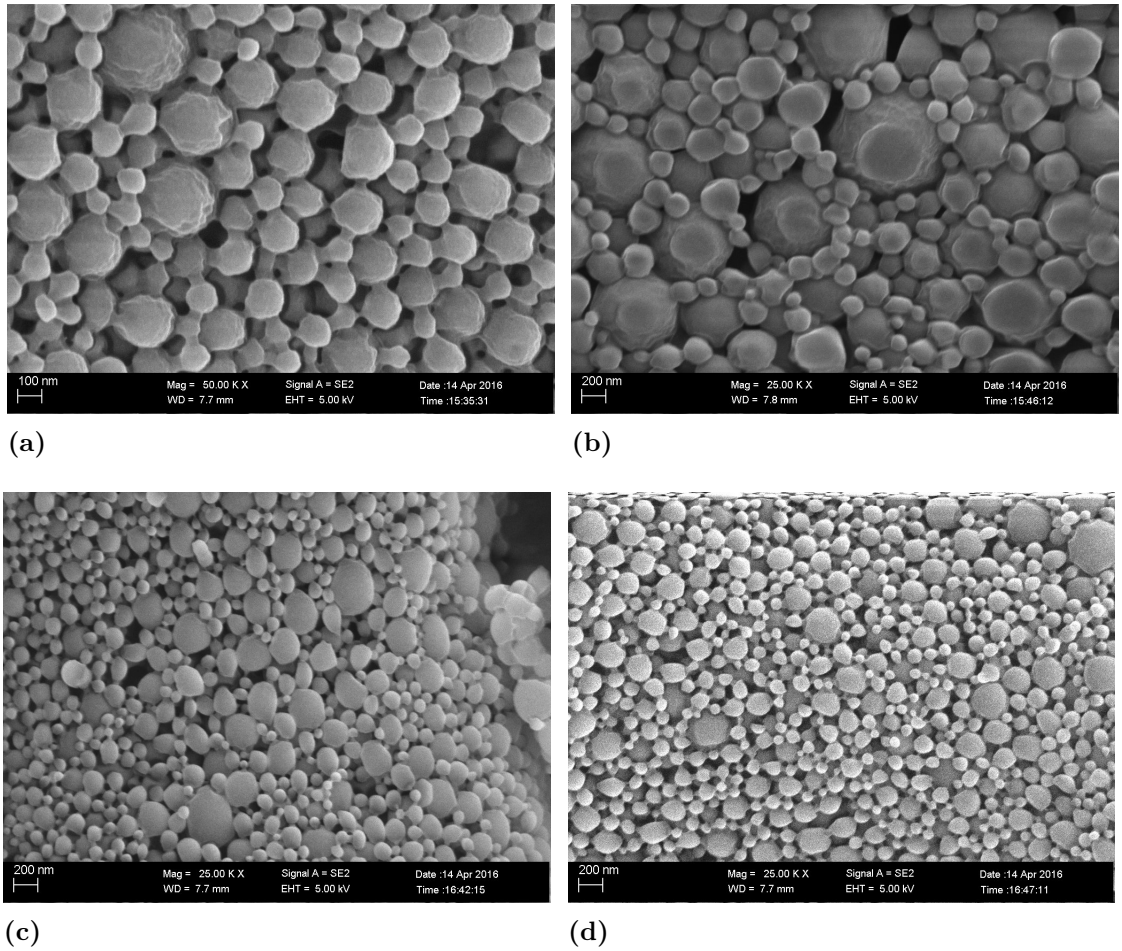
The samples were characterized using a scanning electron microscope (SEM). The sample needs to be conductive and a thin layer of metal is therefore applied before measurements. Two different coatings were tested: gold, see figure 4.1 (a), and platinum, see figure 4.1 (b). Platinum coating displayed distinct particles while gold sputtering seem to melt the sample.



(a) **(b)**

Figure 4.1: (a)Sample 05042016, coated with gold (b)Same sample coated with platinum
Magnification varies

Platinum coating was used henceforth and synthesis parameters were investigated. The sample in figure 4.1 (b) had a polyvinyl alcohol (PVA) concentration of 5% while the sample in figure 4.2 (a) had a PVA concentration of 1%. The sample with lower stabilizer concentration showed slightly bigger and more polydispersed particles, suggesting that a high concentrations of stabilizer facilitates the production of small monodispersed nanoparticles. Another stabilizer, vitamin E TPGS (d-α-tocopherol polyethylene glycol succinate), was tested instead of PVA and the result can be seen in figure 4.2 (b). The particles became more deformed and less monodispersed, and there seemed to be a distinct size gap where small particles surrounded bigger ones. Two samples using different solvents (ethyl acetate and dichloromethane) and different molecular weights of PLGA (20 kDa and 70-88 kDa respectively) were compared with no visual differences, see figure 4.2 (c) and (d) respectively.



(a) **(b)**

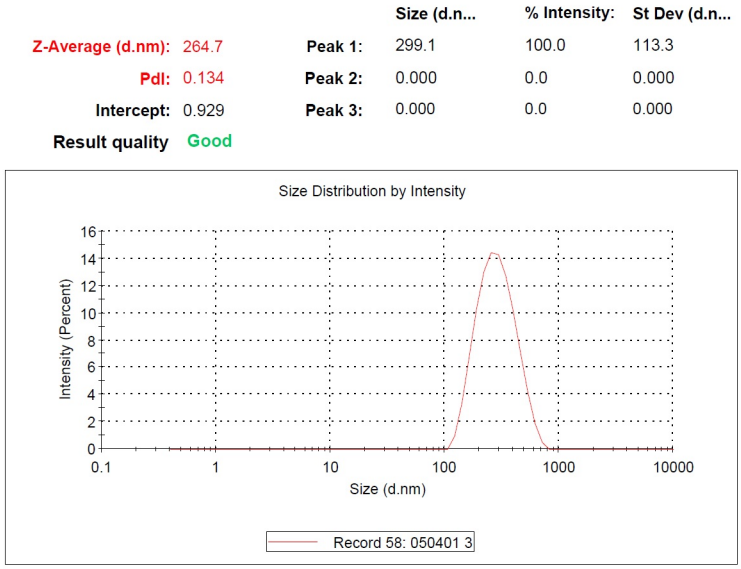
(c) **(d)**

Figure 4.2: (a) Sample 06042016a (1% PVA) (b)06042016b (Vitamin E TPGS) (c)23032016a (Ethyl acetate, PLGA: 20 kDa) (d)23032016b (Dichloromethane PLGA:70-88 kDa)
Magnification: 25k x

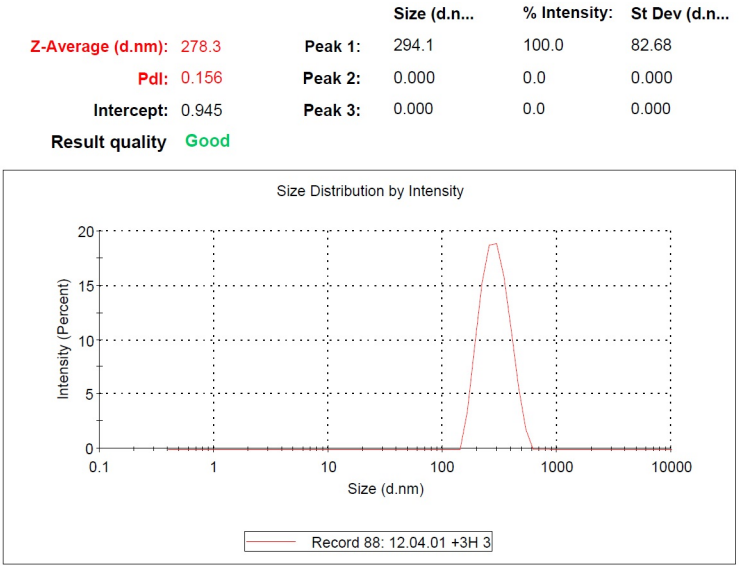
Two samples were synthesized with different sonication settings, 400 W for sample 26052016a and 50 W for sample 26052016b. Dynamic light scattering (DLS)

measurements, not showed here, showed that the hydrodynamic diameter was approximately 200 nm for sample 26052016a and 220 nm for 26052016b, a quite small difference.

A repetition of sample 05042016, seen in figure 4.1 (b), was made following identical protocols to test the reproducibility of the synthesis method. The DLS measurements of both samples can be seen in figure 4.3. The samples appear to be of identical size. SEM images of the samples, not shown here, suggested the same. This indicates that the solvent evaporation technique is fairly consistent.



(a)



(b)

Figure 4.3: (a)DLS measurement of sample 05042016, (b) and its repetition, sample 12042016a

An attempt to produce particles of different size was performed. Sample 09062016a, meant to produce small particles, was synthesized with a sonication power of 500 W and 5% PVA concentration while sample 09062016b had 50 W and 1% PVA. DLS measurements showed that the hydrodynamic diameter was approximately 250 nm and 350 nm for sample 09062016a and 09062016b respectively. A comparison of the two samples can be seen in figure 4.4 where a big overlap in size distribution can be observed. There was a big difference in particle yield between the two samples, 6.1% for sample 09062016a and 46% for 09062016b, an indication that the yield might depend on particle size. Sample 09062016b possessed desired morphology and sufficient yields and was therefore used in the *in vitro* tests.

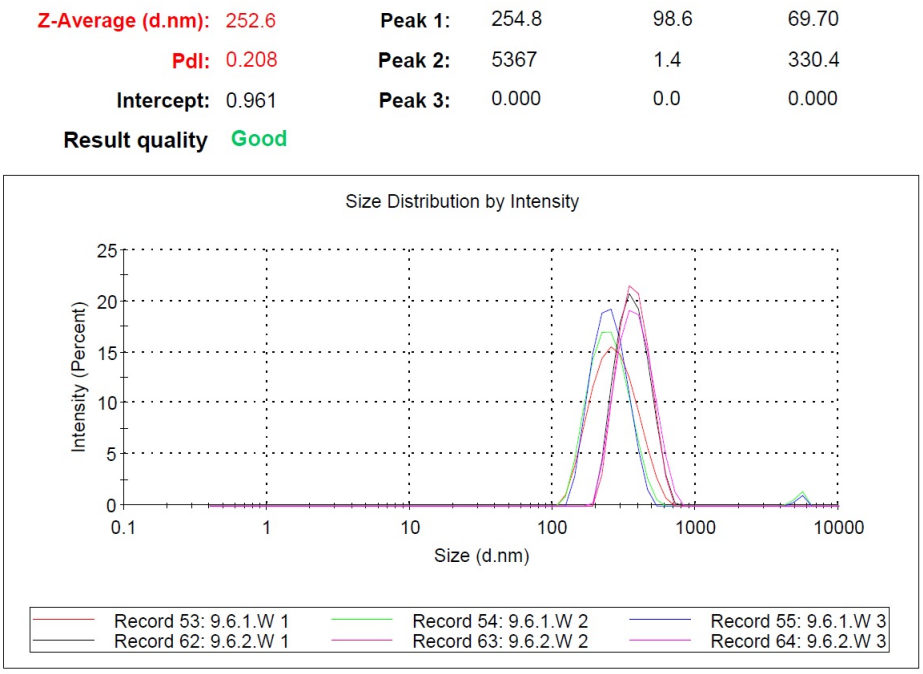
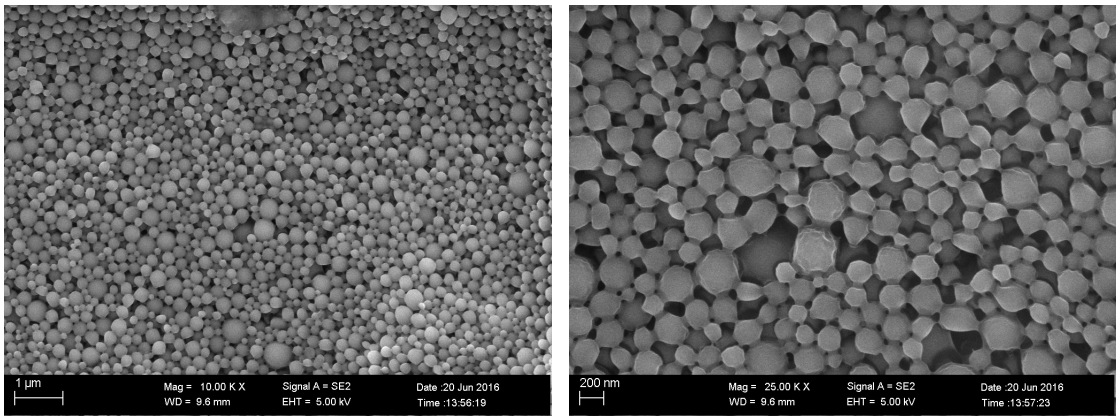


Figure 4.4: DLS measurement of sample 09062016a and 09062016b in the same graphs displays a big overlap in size distributions.

4.2 Nanoparticle characterization

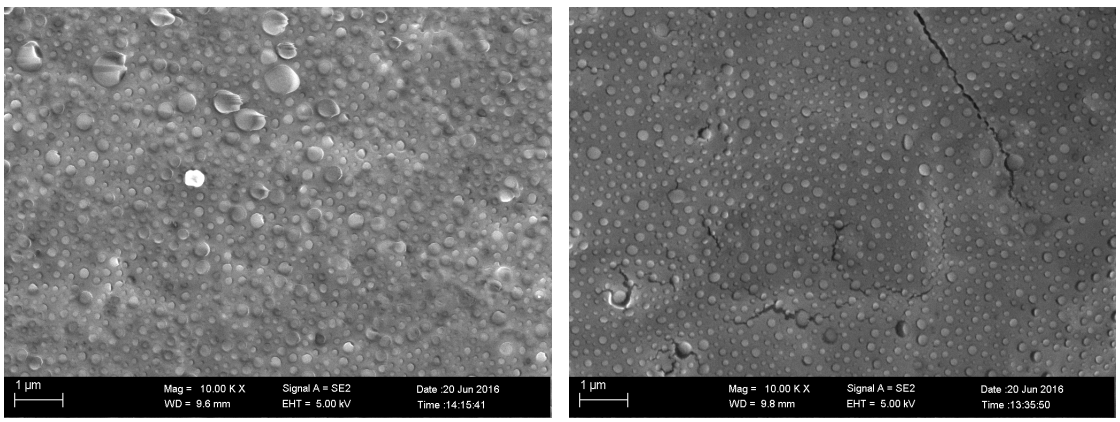
The PLGA nanoparticles used in the *in vitro* tests, sample 09062016b, were qualitative analyzed with an electron microscopy (SEM), see figure 4.5. As can be seen from figure 4.5 (a), the particles are spherical and approximately of the same size i.e. monodispersed. From figure 4.5 (b) an estimate of the particle size can be made. The particles seem to have a mean diameter of approximately 200 nm, perhaps even less than that. Another observation from the same figure is that the particles are not completely spherical and that some interconnectivity is still present.



(a) **(b)**

Figure 4.5: (a)Sample 09062016b, magnification: 10k x (b)Sample 09062016b, magnification: 25k x

Figure 4.6 shows SEM images of the particles together with trehalose, a sugar that is added as a cryoprotectant for storing. The particles in 4.6 (a) have not been sterilized while the particles in figure 4.6 (b) have been objected to γ-radiation. From the images it is clear that trehalose works as intended and inhibits aggregation by encapsulating the particles. The major difference between the two images is the slight distortion of figure 4.6 (a) which originates from image capturing rather than the sample itself. However cracks can be seen in figure 4.6 (b) which could have been caused by the sterilization. These cracks seem to only affect the cryoprotectant and leave the particles unharmed.



(a) **(b)**

Figure 4.6: (a)Sample 09062016b with trehalose (b)Sterilized sample 09062016b with trehalose
Magnification 10k x

DLS was used to obtain quantitative information of particle size and size distribution. An example of how the results looked like can be seen in figure 4.7. The figure depicts three DLS measurements of PLGA nanoparticles in water. Z-average

diameter is the overall average size and peak diameter is the average size for each peak, and their values are 357 nm and 385 nm respectively. The fact that these two numbers are relatively close suggests monodispersity. A PdI value of 0.079 further supports this is. PdI is an abbreviation for polydispersity index and is an estimation of the size distribution. A value of 0.00 means that all particles have the same size and are therefore completely monodisperse. On the other end of the scale is 1.00 where no particles share the same size and complete polydispersity is the state. The particles appear to have a diameter of approximately 360 nm which correlates fairly well to the SEM images since DLS measures the hydrodynamic diameter.

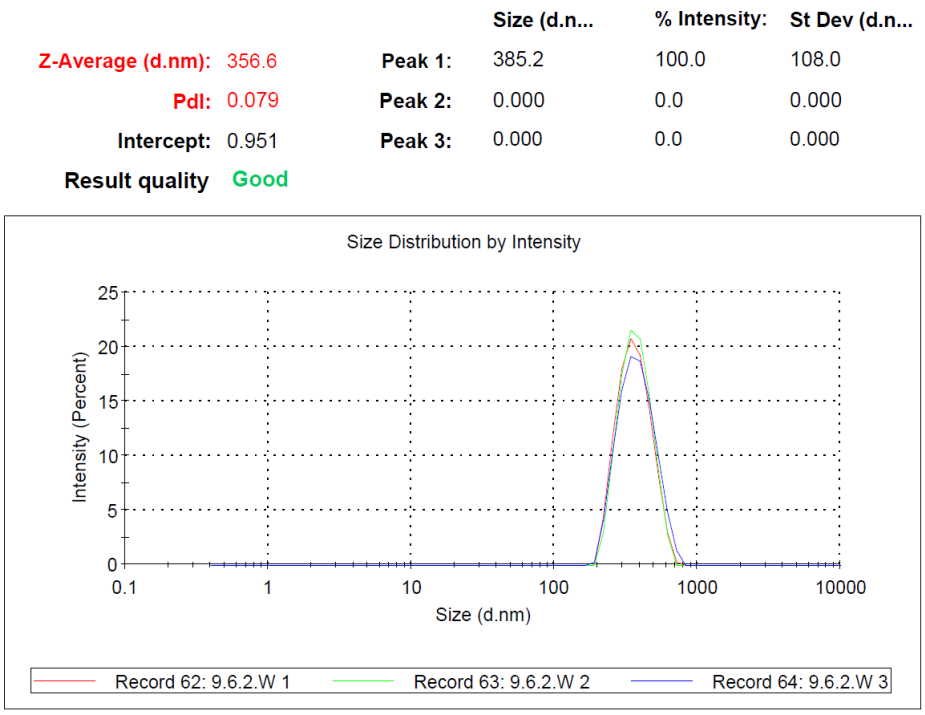


Figure 4.7: DLS results of non-sterilized sample 09062016b in water

Further DLS measurements were performed in cell medium (DMEM LG), cell medium with the addition of serum (FBS) and with γ-sterilized particles in water. All DLS results are summarized in table 4.1. Cell medium was used to simulate the hydrodynamic size of the particles when introduced to the cells. The particle size was slightly increased compared to water. Cell medium together with FBS, a common serum-supplement with an abundance of proteins, was also tested with the working hypothesis that the proteins might attach to the particle surface and form a corona which should increase of the hydrodynamic diameter. This showed to not be the case since no change in size could be observed with the addition of FBS. However the polydispersity index decreased, suggesting that the proteins might stabilize the particles and reduce particle agglomeration. Finally particles sterilized with γ-radiation were tested and they proved to be both bigger and less monodisperse.

Table 4.1: Summary of DLS measurements in different mediums

	Size (nm)	PDI
Water	357	0.079
Cell medium	372	0.103
Cell medium + FBS	372	0.053
Water (sterilized NPs)	421	0.233

Figure 4.8 shows the ζ-potential of the sterilized particles. ζ-potential is the electric potential of the slipping plane and high values are generally desired to obtain stable colloids through electrostatic repulsion. As showed in figure 4.8 the ζ-potential of the particles is -2.92, a relatively low number.

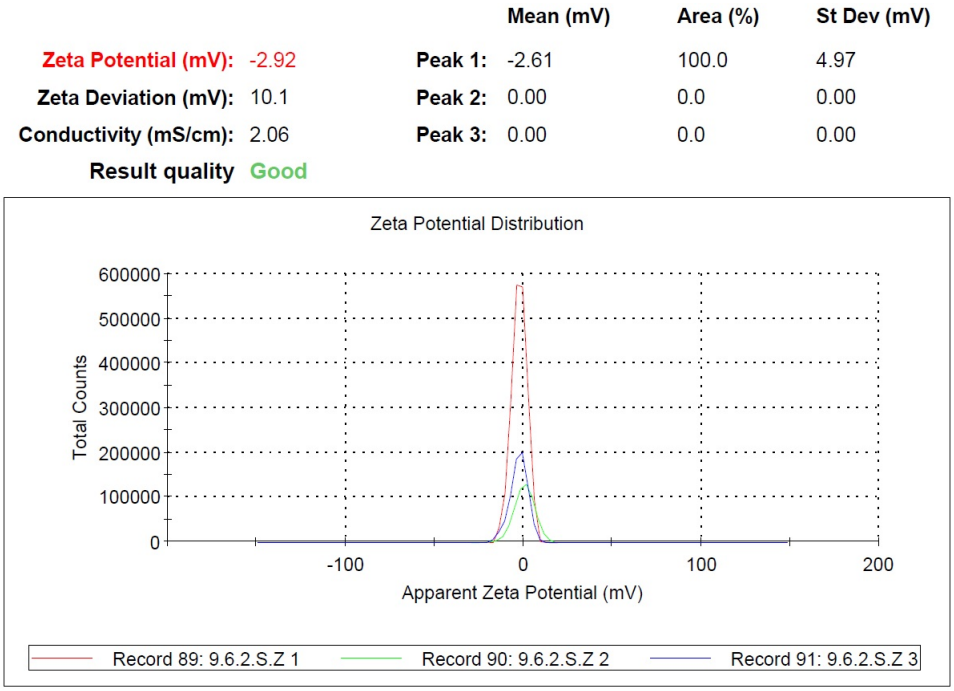


Figure 4.8: ζ-potential of sample 09062016b in water

4.3 Cell viability

A CCK-8 test was performed to investigate the viability of the cells when exposed to different concentrations of the PLGA nanoparticles. The result can be seen in figure 4.9 where the bars have been normalized to the control of each day having a value of one. An interpretation of the result is that the cell viability is not immediately affected but is decreased at day 3. The trend then shifts as the cells get accustomed to the particles and the viability is almost on par with the control after a week. However, big standard deviations make the result unreliable.

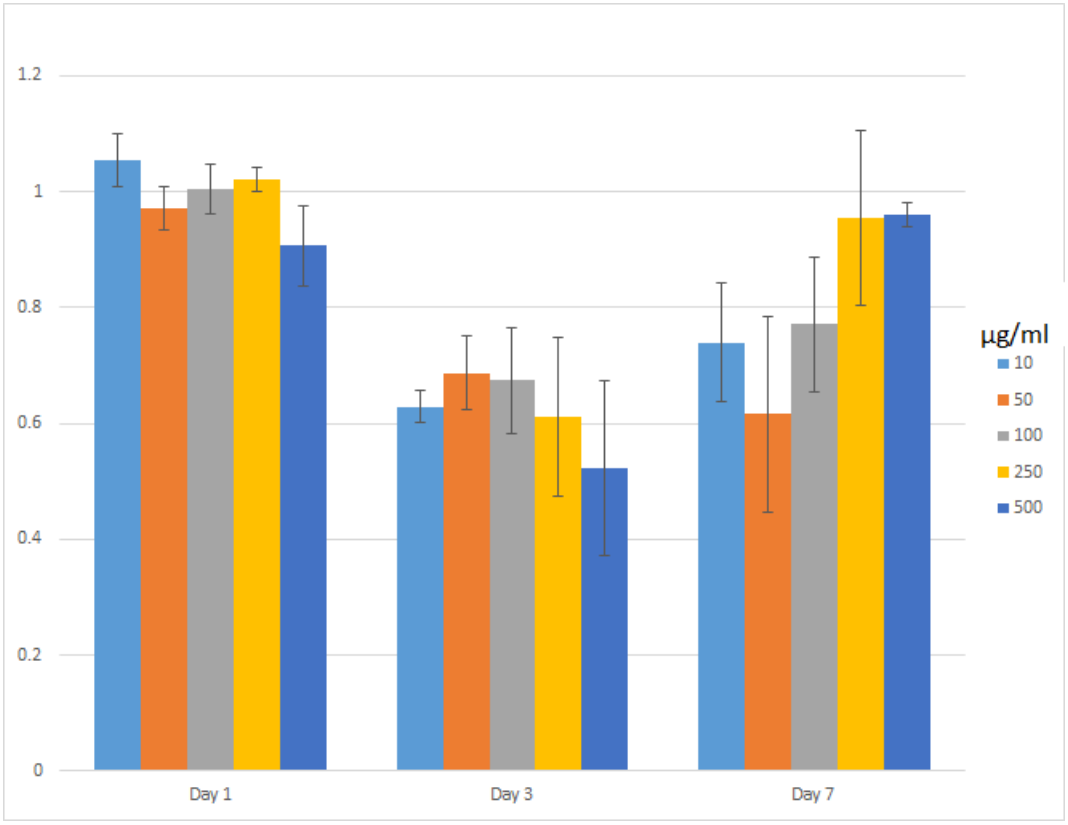


Figure 4.9: Cell viability test (CCK-8) of hMSCs after introduction of different concentrations of PLGA nanoparticles.

Paired Student's t-tests was performed to investigate if the deviations from the control were significant. The null hypothesis was that there was no difference in viability between the control and the cells objected to PLGA nanoparticles and the α-value was set to 0.05. The null hypothesis was deemed correct for all concentrations on Day 1 since no sample deviated much from the control. On contrast the null hypothesis had to be discarded for all samples on Day 3. Interestingly on Day 7 the two samples with the highest particle concentrations (250 and 500 μg/ml) were not significantly different from the control while the 50 and 100 μg/ml samples were. Even so, sample 500 μg/ml was not significantly different from sample 100 μg/ml. It was however significantly different from sample 50 μg/ml. The sample with the lowest particle concentration (10 μg/ml) broke the trend and was not significantly different from the control, but it had one less data point which has a negative effect on the quality of the t-test.

4.4 Osteogenic differentiation

Alkaline phosphatase (ALP) is highly expressed in mineralized tissue cells and can therefore act as an osteogenic marker. In figure 4.10 differentiated and non-differentiated hMSCs objected to varying concentration of PLGA nanoparticles have been ALP-stained. No difference could be observed between the samples.

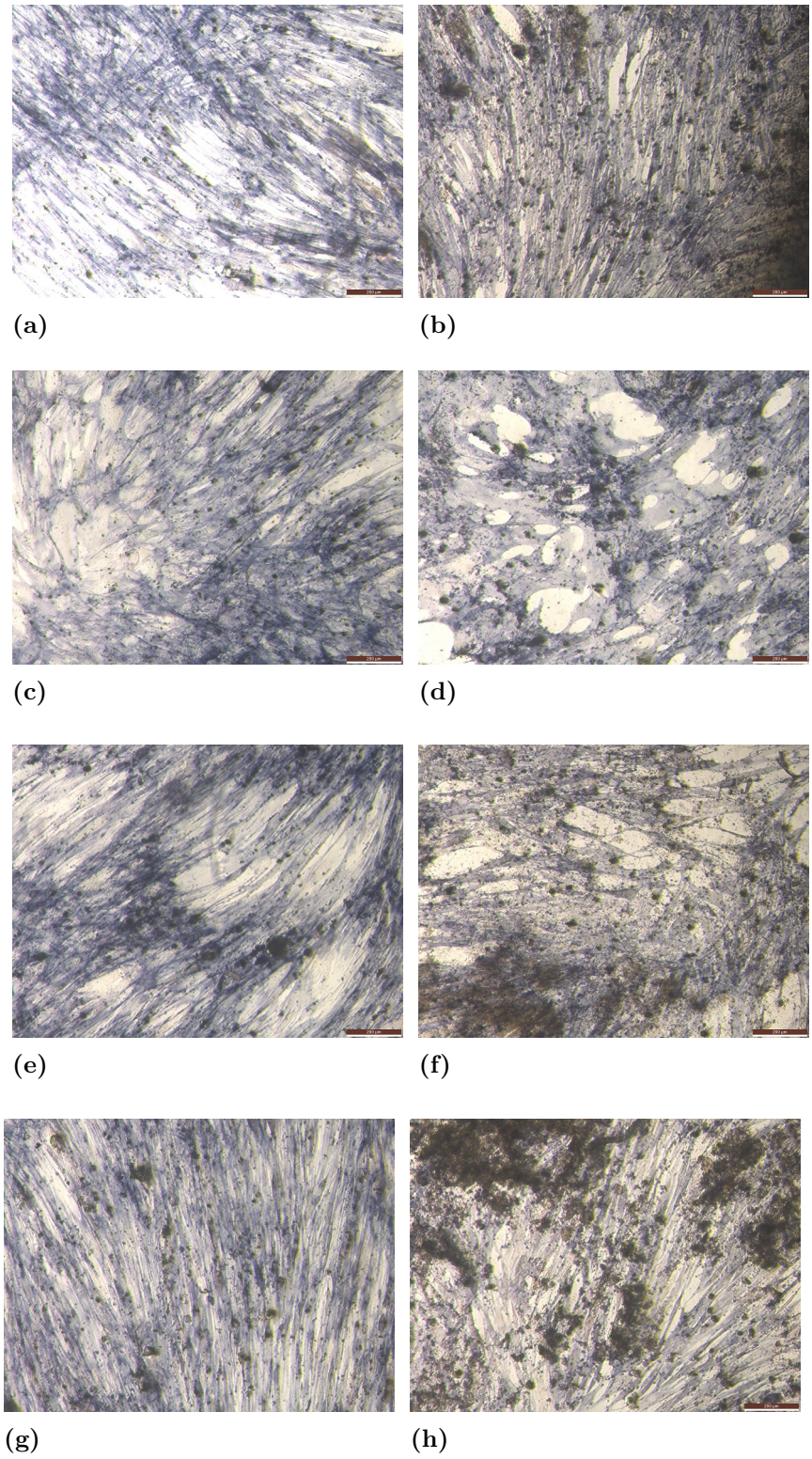


Figure 4.10: ALP stained hMSCs objected to different concentration of PLGA nanoparticles.
Left column: non-differentiated cells, right column: osteogenic differentiated cells
(a)(b)Control (c)(d)10 μg/ml (e)(f)100 μg/ml (g)(h)500 μg/ml
Size of bar: 200 μm

Osteoblasts deposit calcium into the extra cellular matrix during bone formation. The calcium can be stained with Alizarin Red S, abbreviated ARS, and can thus function as an osteogenic marker. Differentiated and non-differentiated hMCSs objected to varying concentrations of PLGA nanoparticles were stained with Alizarin Red S and the result is displayed in figure 4.11. The wells of the left side of the plate contain cells where osteogenic differentiation has been induced while the right side shows non-differentiated cells. The particle concentrations are depicted row-wise and go from top to bottom in the following order: 10, 50, 100, 250, 500 μg/ml with the bottom row as the control with no particles. All differentiated cells were stained while little to no red color could be seen on the non-differentiated cells, a strong indication that the osteogenic differentiation was successful. Another observation is that samples with higher particle concentrations seem to be more stained, which suggests that PLGA nanoparticles indeed promote calcium deposits and perhaps also osteogenesis.

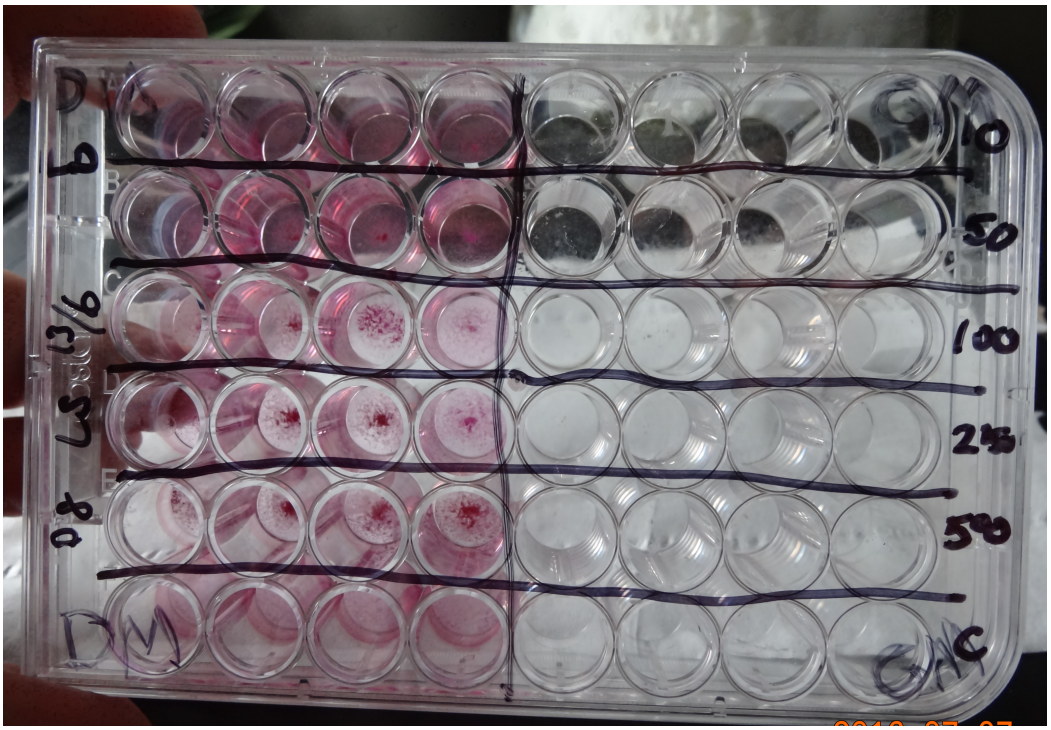


Figure 4.11: Alizarin Red S stained hMSCs.
Left side: osteogenic differentiated cells. Right side: non-differentiated cells.
PLGA nanoparticles concentration row-wise from top to bottom: 10, 50, 100, 250, 500 and 0 μg/ml

Figure 4.12 shows how the ARS-stained cells in 20x magnification. The left column displays non-differentiated cells while the right column contains differentiated cells. Just as in figure 4.11 the differentiated cells are significantly more stained. It is not entirely clear whether the black dots are calcium nodes or particle agglomerates, but the absence of dots in the non-differentiated cells is an indication of the former.

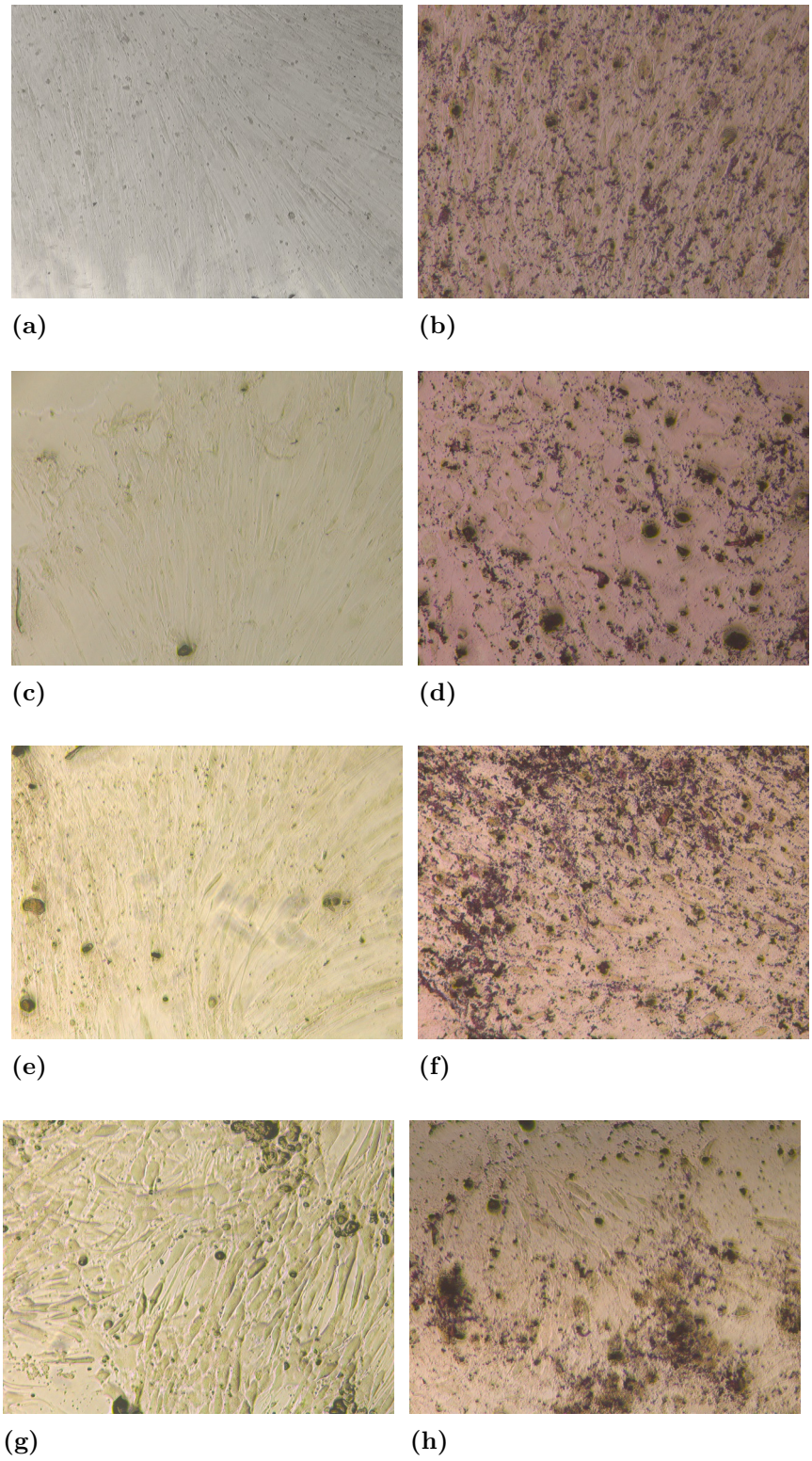


Figure 4.12: Alizarin red S stained hMSCs objected to different concentration of PLGA nanoparticles.
Left column: non-differentiated cells, right column: osteogenic differentiated cells
(a)(b)Control (c)(d)10 μg/ml (e)(f)100 μg/ml (g)(h) 500μg/ml
Magnification: 20 x

The OD value of the samples stained with ARS was measured with a spectrophotometer to obtain quantitative information of the calcium levels. The result can be seen in figure 4.13. As before it was clear that the differentiated cells deposited far more extracellular calcium which strongly suggests a successful osteogenic differentiation. Surprisingly the result indicated that calcium deposits decreased with increased concentrations of particles, which is the opposite trend showed in figure 4.11.

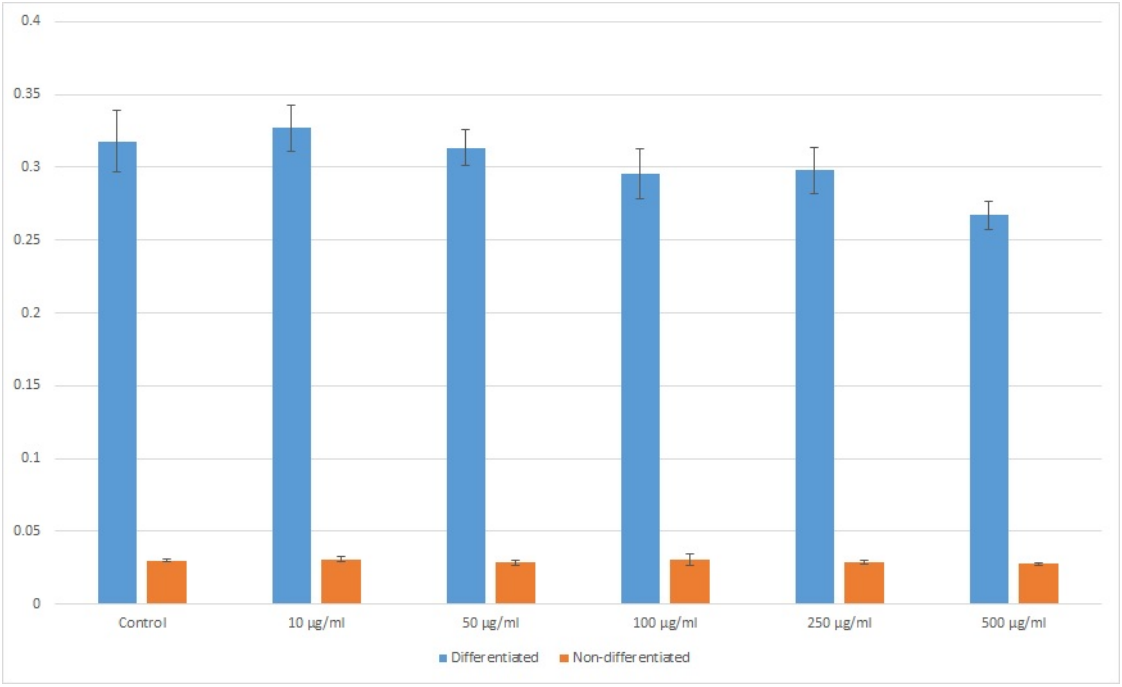


Figure 4.13: OD value after Alizarin Red S staining of hMSCs objected to different concentrations of PLGA nanoparticles.

A paired student's t-test with an α-value of 0.05 was performed on the data displayed in figure 4.13 to conclude whether differences in absorbance of the differentiated cells was significant or not. Only the cells objected to the highest concentration of PLGA particles, 500 μg/ml, displayed significantly different absorbance levels compared to the control. There was also a significant difference between 500 mg/ml and 250 μg/ml.

5
Discussion

In this study, only the molecular weight and the mer ratio were known for the PLGA used. Stereochemistry, tacticity and crystallinity were unknown properties. This lack of information had no major impact in this study due to the limited investigation of the particle synthesis. Further work should however include the complete properties of PLGA to improve interpretation of results and facilitate comparison between different studies.

PLGA nanoparticles have attracted considerable attention in the field of drug delivery. This topic has been addressed in the section 2.1.5 but not utilized in the method. The reason is that although the particles should not include a drug in order to investigate the effect of pure PLGA, a fluorescent marker (namely fluorescein isothiocyanate, FITC) was planned to be encapsulated to visualize cellular uptake. A theoretical background of drug release behavior was therefore suiting. However the internalization test was discarded due to lack of time and no marker was used.

The solvent evaporation method has showed to be a straightforward, efficient and accessible technique to produce PLGA nanoparticles. The continuity of the method is acceptable since the results of two different batches showed just slight differences. However this was just one test and the matter of reproducibility should to be investigated further. It was challenging to produce particles with significant size difference, and overlaps in size distribution were always observed. This might render the solvent evaporation method unsuitable when a fine control of particle size is desired.

The size of the particles seemed to affect the yield of the synthesis. A drastic increase in yield was observed when the particle size was increased from 250 nm to 350 nm. Based on this a rough estimate is that there is a relatively sharp threshold of around 300 nm in particle size where the particle collection step fails to maintain the particles. Smaller particles are less affected by the centrifugation and ends up in the supernatant and are washed away. This could be avoided by increasing the centrifugation speed, but that caused the pellet to be very dense and close to impossible to resuspend. Other particle collection methods need to be utilized if particles $\leq$300 nm are to be produced with desirable yields with the solvent evaporation technique.

It proved challenging to control morphology of the PLGA nanoparticles. The effect of each synthesis parameter on particle size, shape and polydispersity should be investigated by executing a test matrix where one parameter at the time is varied. Factorial design could be implemented as well to examine how the parameters

depend on each other. This could unfortunately not be performed in this project due to time limits and technical problems. However from the results obtained some trends could be observed. Polyvinyl alcohol (PVA) proved to be a more efficient stabilizer compared to vitamin E TPGS. PVA concentration seemed to have the biggest impact on particle size and polydispersity. A high amount of stabilizer maintains the emulsion and allows for smaller particles. This could pose a problem since it is difficult to wash away high concentrations of PVA which might render the particles cytotoxic to the cells. However no indications of these complications could be observed during the project. Sonication settings, molecular weight of PLGA and organic solvent seemed to have a minor impact in the synthesis.

The SEM images seen in figure 4.5 displays relative monodispersed particles even though some particles appears to be very small, around 50 nm. The particles, especially the smaller ones, can be seen to be interconnected. It is unlikely that the interconnectivity observed is platinum bridges since the thickness of the conducting coating layer is normally just around 10 nm. A more plausible explanation is that some heat transfer occurred during the metal sputtering which caused the connecting particles to melt together. The fact that PLGA is a heat sensitive polymer and the drastic change of results when platinum coating was used in favor for gold suggest that the coating procedure is important and that there is room for improvement. Perhaps carbon coating would be more suitable for small PLGA nanoparticles. The particles are not completely spherical but more rock-like, a phenomena more pronounced in smaller particles. It has already been established that the metal coating might deform the particles, however melting would smooth out the surface rather than creating sharp angles. It is unclear when the particles get this shape but some deformation might occur during the particle collection step when the sample is centrifuged.

Trehalose seems to be an excellent cryoprotectant. From observing figure 4.6 it can be clearly seen that trehalose encapsulates each particle and thus inhibiting aggregation. The sugar will dissolve in an aqueous solution, like cell medium, and release the non-aggregated particles. Trehalose is non-toxic and should not have any adverse effects on the cells.

No significant effect of sterilization through γ-irradiation on particle morphology can be seen when observing figure 4.6. It has no visible effect on the cryoprotectant trehalose either. An image of sterilized sample without trehalose would have been good for comparison but was unfortunately not available. DLS measurements indicate that both particle size and size distribution increase slightly after sterilization. This is expected since γ-rays degrade the polymer chain which facilitates absorption of water and thus results in swelling of the particle. Even though γ-irradiation causes deformations it is currently the most suiting technique for sterilization of PLGA nanoparticles for medical studies since autoclavation quickly degrades the polymer and ethylene oxide leaves toxic residues.

The hydrodynamic diameter of the particles was slightly bigger in cell medium com-

pared to water according to DLS measurements, see table 4.1. The difference is difficult to explain because cell medium contains more ions than water and should therefore be more efficient in reducing the thickness of the electron double layer, also known as the *Debye length*, of the particles. A Debye length is the distance at which the electrostatic effects cease to affect the suspension. Ions shield the charge of the particles and therefore decrease the Debye length which in turn allows for faster diffusion and an apparent decrease in hydrodynamic diameter. At first it was theorized that the cell medium possessed a higher viscosity. If so the particles would diffuse slower in cell medium compared to water and thus appear to have a bigger hydrodynamic diameter. However this is probably not the case since the viscosity of cell medium and water is more or less identical. A more plausible explanation is that the size difference is very small, only 15 nm, and therefore probably within the standard deviation. A bigger size distribution can also be observed for cell medium. This could be an effect of the increased concentration of ions which shield the surface charge, a property that stabilizes the particle through electrostatic repulsion. High concentration of ions can therefore increase the risk of particle agglomeration. However, the ζ-potential was already low which decreases the impact of electrolytes.

Cell medium together with FBS was tested as well to investigate if the proteins of the serum would form a corona around the particles. The particle size remained the same as with protein-free medium. This is not completely unexpected because even though a protein corona intuitively should increase the particle size the enlargement is probably insignificant in comparison. The polydispersity index decreased however, which could be an indication that proteins had adsorbed on the polymer surface and stabilized the particle through steric hindrance (stabilization through electrostatic repulsion is unlikely due to the high concentration of ions).

Unfortunately no DLS measurements were performed on sterilized particles in cell medium with FBS, which would be the environment most similar to the one in the *in vitro* tests. In lack of this test it can be discussed whether the DLS results from non-sterilized particles in cell medium with FBS or sterilized particles in water is the most appropriate. It is important to have in mind that the difference between the two samples is relatively small and that none of them are exact. DLS estimates the hydrodynamic diameter of the particles and not the actual size. For the measurements to be exact the particles need to be a hard perfect spheres, and PLGA nanoparticles are neither. DLS measurements are still an useful tool to get an estimate of the size.

The ζ-potential of the particles was very low meaning that the particles are almost without surface charge. PLGA nanoparticles is normally expected to have a high negative ζ-potential at neutral pH due to the anionic carboxyl groups of the polymer. Why the measured values were close to zero could be explained by the high concentration of PVA used. Residuals of the stabilizer might shield the carboxyl groups and thus decreasing the ζ-potential. This affects the stability of the particles because attractive forces (e.g. van der Waals and hydrophobic forces) might overcome the weak electrostatic repulsion and cause particle agglomeration. Cellular

uptake could also affected.

The results from the cell viability test seen in figure 4.9 are difficult to interpret due to high standard deviation. The results implies that the introduction of the PLGA nanoparticles has a negative impact on the viability and/or proliferation of the cells. This could be because of the polymer itself, the size of the particles or remaining residues of emulsifier (PVA). This seems to be independent of concentration which is surprising. After a week the cells seem to recover and cell viability is almost at normal levels. This could be because the cells have acclimatized to the presence of the particles or that the particles have been washed away with the changes of cell medium. The samples with the highest particle concentrations seem to be first in reaching viability on pair with the control, which was unexpected. It is important to have in mind that the results depicted in figure 4.9 are normalized with the control of each day and not by the control from the first day. This allows for comparison with the control for each day, but not for comparing the days. As stated earlier the big variances make it difficult to make any direct conclusions. The main interpretations of the result is that the PLGA nanoparticles might be slightly cytotoxic for the cells in the the short run but not after one week after introduction and that the particle concentration have no significant impact on cell viability.

ALP staining of non-differentiated hMSCs and hMSCs where osteogenic differentiation had been induced showed no difference, as can be seen in figure 4.10. Particle concentration did not affect the result either. There are several possible explanations for this, one being that the osteogenic differentiation had been unsuccessful. This is unlikely since staining of extra cellular deposits of calcium proved successful differentiation. Given that alkaline phosphatase is present in most cells one explanation could be that the expressed levels were indistinguishable from the control due to over-staining. Another explanation is, since alkaline phosphatase is an early osteogenic marker, that the peak of protein expression was before the staining and the expression was therefore back on normal level. A quantitative analysis might have given some insight in the matter but was unfortunately not performed due to lack of time and knowledge.

The staining of extracellular calcium deposits showed a distinct difference between differentiated and non-differentiated cells, as seen in figure 4.11, which strongly indicated that the osteogenic differentiation of hMSCs was successful. There was also an interesting trend that the amount of stained calcium correlated to the particle concentration. This could be confirmed upon visual inspection with microscope, see figure 4.12. Black dots was observed and the amount seemed to increase with higher particle concentrations. It is unclear if they were spots of high calcium concentrations or remains of the nanoparticles. They seemed to not be as abundant for the non-differentiated cells but were still present- However, it is important to have in mind that these results are qualitative and only displays a small portion of the sample. Quantitative measurements, figure 4.13, indicated a trend where the amount of calcium had an inverse correlation with particle concentration which contradicted the first hypothesis. This was significantly verified only with sample with

the highest particle concentration.

An explanation for these contradicting results could be that nanoparticles were still present in the wells and had agglomerated. The dye might have adsorbed to the particle agglomerates and thus given the impression that calcium was more abundant with increased particle concentrations. The optical properties of the nanoparticles are unknown and could have a higher impact in the analysis by the spectrophotometer than the dye. Factors in favor for this hypothesis is that the cells were never thoroughly washed after particle incubation since it was assumed that free particles would be removed during medium change. Additionally, white residues were visible to the naked eye on the non-differentiated wells of figure 4.11 and seemed to increase in extent with higher particle concentrations, which could be particle agglomerates. How Alizarin Red S could adsorb to the particles is difficult to explain since they dye normally binds to dications as Ca^{2+} and PLGA surfaces normally are negatively charged. The Alizarin Red S staining offers more questions than it answers. The result could be an interpreted as PLGA enhance osteogenesis, or at least deposition of extracellular calcium, which makes it a promising candidate for tissue engineering. They could also be interpreted as PLGA nanoparticles have no or even adverse effect on osteogenesis. In either way the experiment needs to be repeated in order to come to any conclusions.

The priority of future work should be to obtain a thorough understanding of the synthesis of PLGA nanoparticles. It is not until a proper control of the synthesis process is achieved that *in vitro* tests become truly meaningful, otherwise it remains unclear exactly what the cells have been objected to and reproducibility is hampered. Synthesis parameters that ought to be investigated further are PLGA concentration, type of organic solvent, type of stabilizer, phase ratios, evaporation time, centrifugation settings and sterilization. In addition the properties of PLGA should be known, such as molecular weight, mer ratio, tacticity, stereochemistry, crystallinity and glass transition temperature. This information facilitates explanation of for example degradation time or drug release kinetics.

When PLGA nanoparticles can be produced satisfactorily *in vitro* tests should be conducted to established whether the particles have an effect on the osteogenic differentiation of hMSCs or not. Staining for alkaline phosphatase and extracellular calcium have been performed in this study but the results were difficult to interpret and the tests should be repeated. Other ways to analyze osteogenesis are to use immunohistochemistry or PCR to measure the expressed levels of protein and RNA respectively of genes associated with osteogenesis, for example genes encoding for collagen I, osteocalcin, osteopontin RUNX2 and alkaline phosphatase.

There is an almost infinite amount of possible future experiments, for example investigating the effect of particle size, charge or drug loading. PLGA nanoparticles seem to have great potential in the medicine, and even though the polymer has been extensively studied for decades there is still much to discover.

6
Conclusion

Monodispersed and spherical PLGA nanoparticles with a diameter of approximately 400 nm have been produced with the single emulsion method. This was a simple and cost efficient method of producing PLGA nanoparticles, but it was difficult to fine tune the morphology of the particles and obtain sufficient yields for particles $\leq$300 nm. This made the single emulsion method not suitable for synthesizing nanoparticles with a very defined size. The concentration of stabilizer proved to be the most significant parameter during the synthesis while sonication power, molecular weight of the polymer and organic solvent had minor impact on the result. Choice of stabilizer affected the result as PVA proved to produce smaller and more monodispersed particles compared to vitamin E TPGS.

Type of metal coating for SEM imaging proved to be crucial since gold sputtering destroyed the sample while platinum produced good results. The reason was most likely heat development that caused the heat sensitive polymer to melt. SEM images indicated that trehalose effectively encapsulates the nanoparticles and inhibits aggregation during freezing. The cryoprotectant seems to not be affected by sterilization by γ-irradiation. The PLGA nanoparticles on the other hand are affected by the sterilization and DLS measurements show swelling and increased polydispersity. No major difference can be observed in size nor polydispersity index when performing DLS measurements in water, cell medium and medium with FBS. The ζ-potential of the particles was very low which could cause particle agglomeration.

The cytotoxicity test proved difficult to interpret but indicated that the particles had an initial negative impact on cell viability. The cells seemed to recover quickly and full viability in comparison with control was reached after a week. Particle concentration had no major impact on the result. In conclusion, PLGA nanoparticles might possess a minor short term cell toxicity. Whether it is caused by the particles or of PVA residues is unclear.

The alkaline phosphatase staining did not provide any proof of osteogenic differentiation. The concentration of particles did not have an impact on these results. The differentiation was probably successful since staining of extracellular calcium deposits had proved it in another test. The conclusion is that the alkaline phosphatase staining was either not performed correctly or executed too late in the osteogenesis when alkaline phosphatase levels had diminished. Quantitative measurements might have provided an insight but were unfortunately not performed.

Staining of extracellular calcium deposits displayed a distinct difference between non-differentiated and differentiated cells, proving that the osteogenic differentiation was successful. Observation with the naked eye suggests that the calcium levels correlate with the particle concentrations while quantitative measurement with spectrophotometer implies the opposite. There are several indications that agglomerates of the particles might still be present outside the cells which could have produced the conflicting results. The final conclusion is that it can not be proven that PLGA nanoparticles induce osteogenesis, but some results suggest it and the topic is therefore interesting for further research.

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A

Appendix 1

A.1 Safety Data Sheet

None of the chemicals below (PLGA, FITC, PVA and DCM) except EtAc have any explosion or fire hazard and any fire can be put out with common extinguishers.

A.1.1 PLGA

PLGA has no dangerous safety hazard. Avoid skin and eye contact (irritating), if contact then wash thoroughly with water and consult a physician. Avoid breathing dust and fumes, if breathed remove to fresh air and consult physician. If ingested, do not induce vomiting and consult physician (Surmodics Pharmaceuticals Inc n.d.). Spill, leakage and release is removed by shovel or sweeping up and transferring it to approved disposal area.

A.1.2 Fluorescein isothiocyanate (FITC)

Acute health effects are harmful if exposed by contact or inhalation. Eye and skin contact is treated by flushing with copious amounts of water for at least 15 minutes, remove exposed clothes and call a physician. If serious, seek medical attention. If inhaled, remove to fresh air (if breathing becomes difficult, consult a physician), (Bioworlds 2006).

A.1.3 Polyvinyl alcohol (PVA)

May cause irritation if exposed to skin, eyes or ingested or inhaled. If in contact, flush with large amounts of water for at least 15 minutes while removing contaminated clothes. If ingested, get medical health and wash mouth out with water. If inhaled, remove from exposure to fresh air. If breathing becomes difficult, get medical aid, (Acros Organics 2005).

A.1.4 Dichloromethane (DCM)

Toxic. Harmful if swallowed (do not induce vomit, wash mouth and call a physician). Irritating to eyes (flush, call physician), skin (flush with soap) and respiratory system (remove to fresh air, call physician if breathing becomes difficult). May cause cancer through absorption of the skin. Target organs are heart (DCM is converted to

CO2 in the body) and central nervous system (possible dizziness, headache, loss of consciousness and death if high concentrations), (Sigma-Aldrich 2004).

A.1.5 Ethyl acetate (EtAc)

Highly flammable, vapour/air mixtures are explosive. Keep free from open flames, sparks and smoking. Extinguish with powder or carbon dioxide. Inhalation could cause cough, dizziness drowsiness, headache., nausea, unconsciousness. Treat with fresh air and rest. Refer for medical attention. May dry the skin and cause pain if contact with eyes. In both cases rinse with plenty of water and consult a doctor. If ingested rinse mouth and drink plenty of water, (The National Institute for Occupational Safety and Health 1997).

B

Appendix 2

B.1 Particle synthesis optimization

This chapter depicts a summary of all the tests that were performed during the process of investigating and optimizing the synthesis of PLGA nanoparticles together with thoughts and ideas. The aim of this section is to give the reader an understanding of how the project progressed; what have been done, why it was done and how it went. The aim of the project was changed several times and the method was fine-tuned. The final method is found in chapter 3 and its outcome in chapter 4. The complete documentation can be found in my physical lab journal.

The project started immediately with practical work in order to get to know the lab and the synthesis method. Not much was know about previous work at this time and the supervisors intentionally refrained of making any inputs, thus giving us free roam to be creative.

The particle synthesis had mainly three aims; control of particle size, achieve monodispersity and formulate a reproducible protocol. Size-control is crucial to obtain properties exhibited in the nanoscale and allows for testing of different sizes. Monodispersity (all particles have the same size) is required to analyze effect of size. A clear protocol facilitates reproducibility which reduces the batch-to-batch differences and allows further researchers to continue with the work. In addition to these three aims the particles should be spherical and independent (non-networked), contain a marker and have no residues of stabilizer that could affect the interaction with the cells.

The double emulsion method (W/O/W) was chosen as a synthesis method since it is common, relatively simple and it allows for encapsulation of hydrophilic drugs and markers. McCall & Sirianni (2013) has done a great job in formulating a clear and easy to follow protocol for the double emulsion method and the protocol used in this thesis is mostly based on it. During the project certain changes were made but the main parts remain intact. Here follows the main protocol used in this project for synthesis of PLGA nanoparticles.

Preparation of PLGA nanoparticles using a water-in-oil-in-water (w/o/w) emulsion solvent evaporation technique:
Polymer solution:
Dissolve 500 mg PLGA in 5 ml chloroform, dichloromethane (DCM) or ethyl acetate (EtAc) to obtain a concentration of 100 mg PLGA/ml. Add the weighted polymer to the organic solvent and mark the solvent level. Then seal the vial with parafilm and stir magnetically over night. If any evaporation had occurred new solvent should be added.
Stabilizer solution
Dissolve 10 g of polyvinyl chloride (PVA) in 200 ml pre-heated (70°C) deionized water to obtain a concentration of 50 mg PVA/ml or 5% w/v (weight/volume). Stir the PVA mixture in oil bath at 70°C until it is completely dissolved. The PVA solution should be filtered through a 0.22 μm filter before usage to avoid aggregates. To facilitate filtering the concentration can be decreased.
Marker
Dissolve 10 mg of the fluorescence marker *fluorescein isothiocyanate* (FITC) in 1 ml anhydrous ethanol (EtOH) through brief vortexing. Cover the solution in metal foil in order to keep the light sensitive FITC active. Store in fridge.
Organic phase
2 ml PLGA solution (100 mg/ml) in chloroform, DCM or EtAc.
Internal phase
100 μl $\text{FITC}_{(EtOH)}$
External phase
4 ml 5% (w/v)PVA_{aq}
Evaporation phase
100 ml PVA_{aq} (0.1% w/v). 2% v/v of isopropyl alcohol can be added to facilitate evaporation of the organic phase.
Protocol
Add the internal phase dropwise to the organic phase while vortexing to form the primary emulsion. Add the emulsion dropwise to the external phase while vortexing and notice the visual changes. Quickly transfer the mixture to an ultrasonicator and sonicate on ice. What time and power to use depends on the apparatus and the desired particle size, but short burst with pauses are recommended in order to avoid generation of heat. Move the vial up and down to ensure even sonication and avoid contact with the probe. Notice the visual change. The emulsion should turn bone white and become opaque. Transfer the double emulsion to the evaporation phase and stir for 3 h. Cover the beaker in metal foil to protect the fluorescence marker and leave the top open to facilitate evaporation of the organic phase. Collect the particles by centrifuging the sample at 12 kRPM (7.2 kG) for 3x10 min at 20°C. Discard the supernatant after each wash and dissolve the pellet in deionized water by vortexing and/or water bath sonication. After the washing add the cryoprotectant trehalose in a weight ratio of 1:2 trehalose:PLGA and vortex briefly. Do not add trehalose to the samples meant for SEM imaging since the disaccharide makes visualization of the particles difficult. Freeze the sample at -80°C for at least two hours. When the sample is frozen uncap the vial and secure lab tissue or other semipermeable membrane across the top of it. Work fast to avoid melting. If melting does

occur refreeze. Quickly transfer the frozen sample to a lyophilizer and freeze dry at -50°C for two days. Store the lyophilized particles in freezer at 80°C.

The very first synthesis, labeled 01032016, produced big deformed particles clearly visible to the naked eye. It was suggested that the sonication power was too low (50 W) to produce a satisfying emulsion. Lack of experience was probably a contributing factor as well. In the second synthesis (sample 02032016) the sonication power was increased considerably from 50 W to 400 W in an attempt to produce smaller particles. Just a few particles were visible after the synthesis, suggesting the particle size indeed had decreased. A colleague had SEM-time the same day and offered to take images of the second sample prior to lyophilization, which can be seen in figure B.1. The particles were big and quite polydispersed, but were otherwise spherical, non-interconnected and close to the nanoscale.

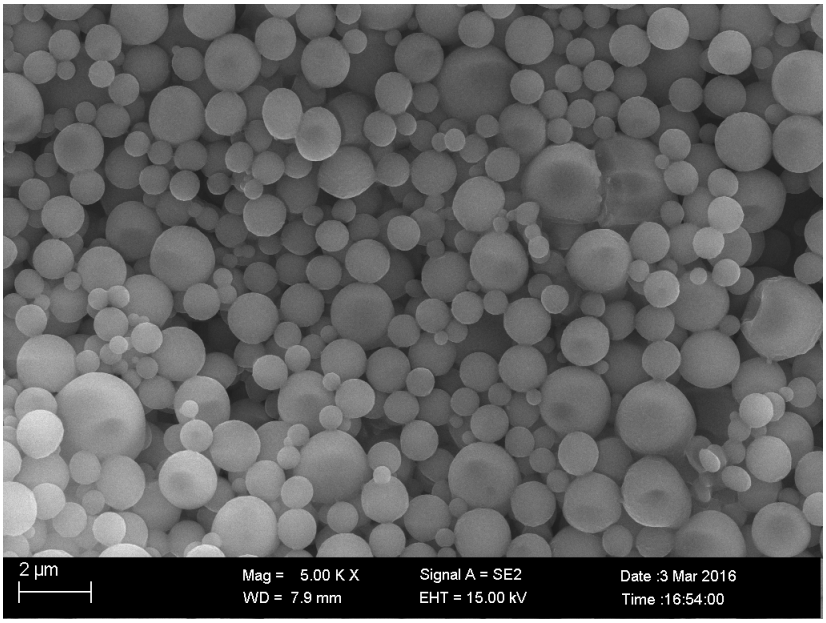


Figure B.1: SEM image of second sample (02032016) produced by lab colleague.

Observing figure B.1 an assumption was made that the synthesis procedure was satisfying and only needed to be optimized to obtain smaller and more monodispersed particles. New particles were prepared using the same protocol but with varying one parameter at the time in order to deduce which ones that were significant for the end result. Parameters tested were polymer concentration, stabilizer (PVA) concentration and phase ratio.

SEM-time was only available every second week. In the meantime the particles were synthesized without any means of verifying if the synthesis was successful or not. Dynamic light scattering (DLS) was an option but since it requires hard perfect spheres for accurate results it was desired to use SEM-images to confirm that the particles indeed were spherical prior to DLS measurements. Indications of a successful synthesis could however be observed with the naked eye. Some of these signs were a lack of visible particles, no sedimentation and a fine powder after

lyophilization. Most samples displayed these qualities. The results from my first own SEM-imaging, see B.2, were therefore surprising and discouraging. All samples had a coral-like appearance and only hints of particles could be observed, suggesting the spheres had melted together.

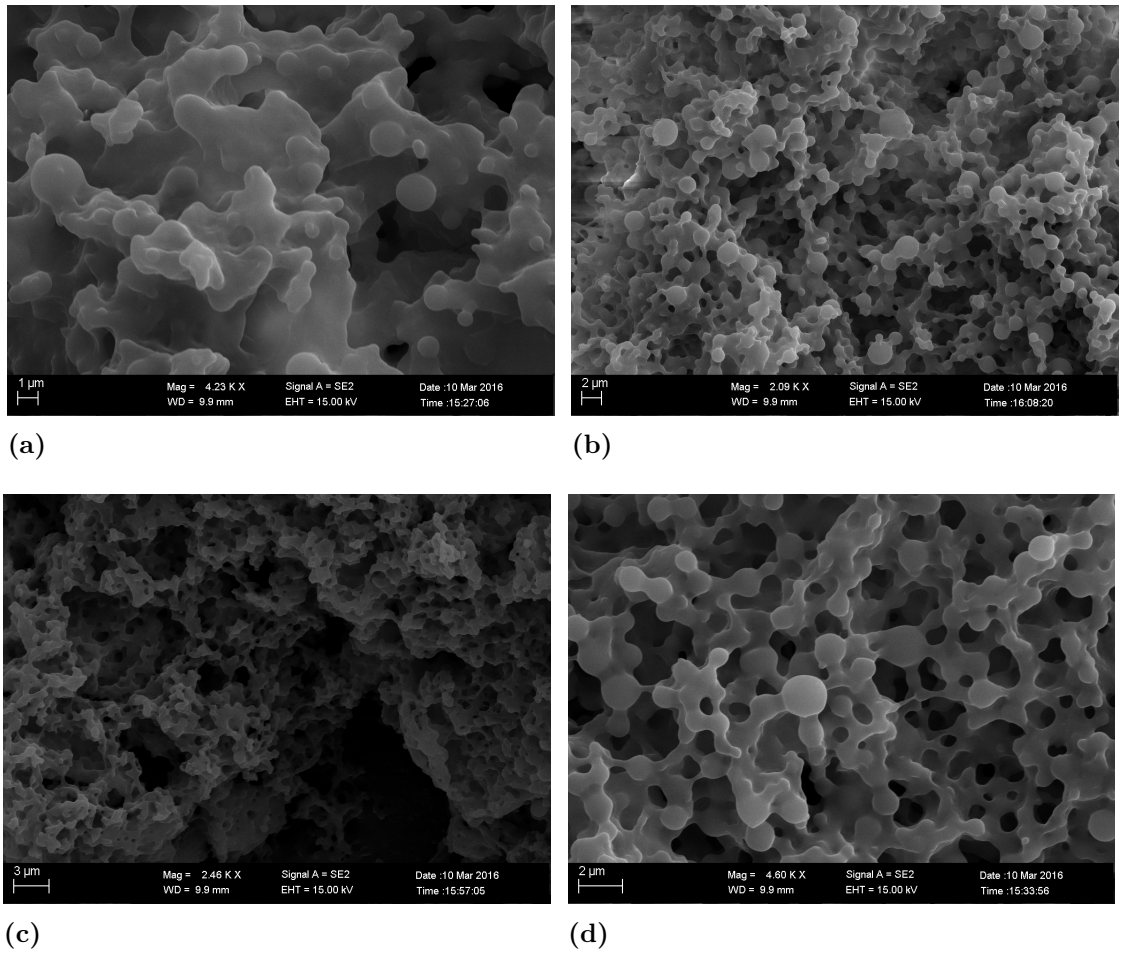


Figure B.2: (a)07032016a (b)07032016b (c)08032016 (d)09032016 Magnifications vary

Lab assistants performed the preparation for SEM-imaging, which involved the metal sputter coating and the installation of the sample in the SEM-apparatus. It was therefore assumed that both figure B.1, produced by the colleague, and B.2, produced by me, had been prepared in identical manner. This assumption proved to be wrong, see later in this chapter, but led to the conclusion that no particles had been formed during the synthesis. Since it appeared that the particles had melted it was proposed that the emulsification step evolved too much heat. It was decided to decrease the sonication time. To ensure emulsification the sonication power was increased. It might sound counterproductive to decrease the time but increase the power, but past samples were subjected to at least 200 W during 10x30s resulting in a total of 60 kWs while the coming experiments should use 700 W in 3x10s with a total of 21 kWs. It was still puzzling why the particles seen in figure B.1 had not

melted even though they had been synthesized with the same sonication settings as them in figure B.2. It was deemed that the successful sample had been a stroke of beginner's luck. In hindsight this was a hasten conclusion without much scientific backing.

In the mean time the particle synthesis continued. Different concentrations of PVA were tested as previous work had stated that stabilizer concentration had a big impact on particle size and size distribution. One observation during prior experiments was the leakage of the fluorescent marker fluorescein isothiocyanate (FITC). The evaporation phase was often stained yellow while the particle powder after lyophilization was completely white, which could be interpreted as all marker had leaked out during the synthesis or that some was still well encapsulated in the particles. Our supervisor suggested to try coupling FITC to (3-aminopropyl) triethoxysilane, more commonly know as APTES. The idea was that the amine group of APTES should bind to the isothiocyanate group of FITC. APTES would then be hydrolyzed in contact with water and bind to PVA through hydrogen bonds. To summarize, APTES would link the marker FITC to the stabilizer PVA which is anchored to the particle surface, see figure B.3.

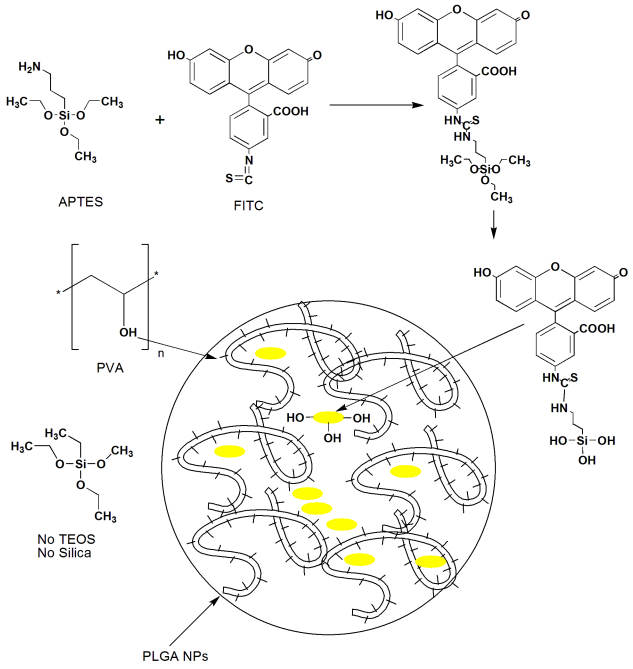
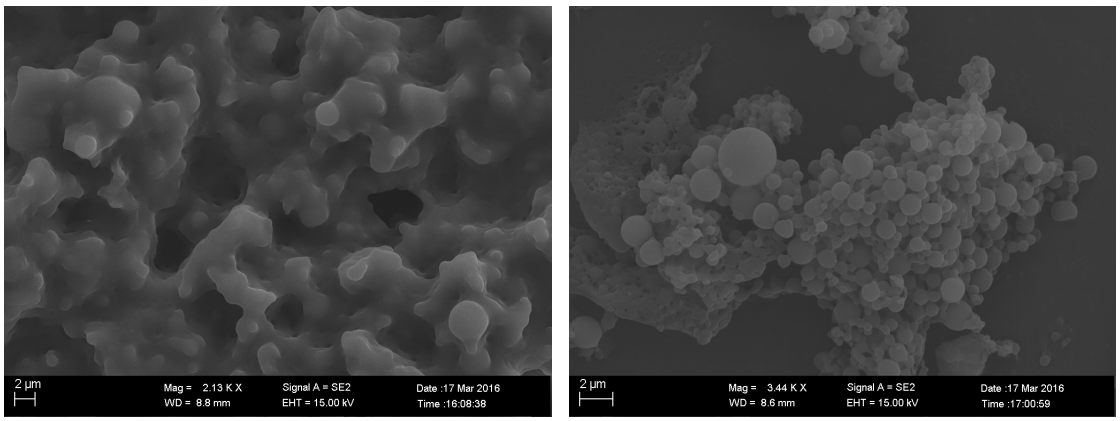


Figure B.3: The amine group of APTES binds to the FITC molecule and then links it to the PLGA particle through hydrogen bonds between the hydrolyzed silanol groups of APTES and the hydroxyl groups of stabilizer PVA.
Source: Tarek Elkhooly

In short, the FITC-APTES solution was prepared as follows. 10 mg FITC was added to an eppendorf tube. 24 μl (4.19 M) APTES was added to 2000 μl anhydrous ethanol and quickly vortexted. Anhydrous ethanol was used to avoid premature hydrolysis of APTES. The APTES solution was transferred to the eppendorf tube containing FITC which was brimmed and sealed to avoid hydrolysis through contact

with air. The lid was parafilmed, covered in metal foil and magnetically stirred over night. Sometimes the preparation of FITC-APTES was unsuccessful and produced visible fibers upon contact with PVA, an indication of premature hydrolysis. When the preparation was successful the samples continue to leak marker. It was decided to not use APTES in further experiments due to the added complexity of the synthesis together with the apparent lack of benefits.

The lyophilized samples were prepared for SEM the same way as the ones in figure B.2, namely by suspend the dry powder in a aliquot of water and some ethanol, mix it in a water bath sonicator followed by evaporation of the liquid phase. By preparing the samples this way an even and thin layer of particles is achieved. However some samples formed a pellet and did not suspend efficiently. Therefore one sample was added dry to the disk used for SEM-imaging in order to test how it affected image quality. Some of the results can be seen in figure B.4



(a) **(b)**

Figure B.4: (a)15032016b, prepared wet. (b)15032016b, prepared dry. Magnifications vary

The result of figure B.4(a) looks almost identical to figure B.2, leading to the conclusion that the sonication power had not been decreased enough or was not the cause for the melting. It could be observed that the sample prepared dry, see figure B.4(b), looks more promising than the other. Although it still appears to have melted, more distinct spheres in the microscale can be observed.

This led to the conclusion that there could be something wrong in the SEM-sample preparation. When consulting the lab colleague who had prepared the first sample seen in figure B.1 it became evident that the sample indeed had been prepared as a dry powder instead of being suspended in water/ethanol. It was hypothesized that the water bath sonication used to suspend the powder evolved too much heat. In hindsight this is not a very plausible explanation since the heat involved in water bath sonication is limited and could also be adjusted, but with the information available it appeared to be a plausible explanation.

Due to the unexpected errors encountered it was desired to limit the scope of the project and streamline the synthesis process. It was therefore decided to try the single emulsion technique. The change would simplify the synthesis and would not have a big impact on the end result since no hydrophilic drug was planned to be encapsulated in the particles. Different solvents for PLGA were tested. Dichloromethane (DCM) was compared to ethyl acetate (EtAc) which is more polar, a property that made it time-consuming to dissolve PLGA but allowed the marker (FITC) to be used directly in the organic phase. FITC was insoluble in DCM and was therefore not used in those samples. These samples were prepared for SEM imaging by applying the dry powder directly instead of suspending it first. Some old samples were tested again using the same technique to see if the if the result changed. All samples, both the newly prepared and the old ones, looked similar with an apparent melting of the particles. The choice of organic solvent did not seem to have a significant impact, but it was difficult to interpret the results since no particles were visible. The most promising image is shown in figure B.5. From the figure it is evident that particles have existed but that they have become deformed and formed a network, probably due to overheating.

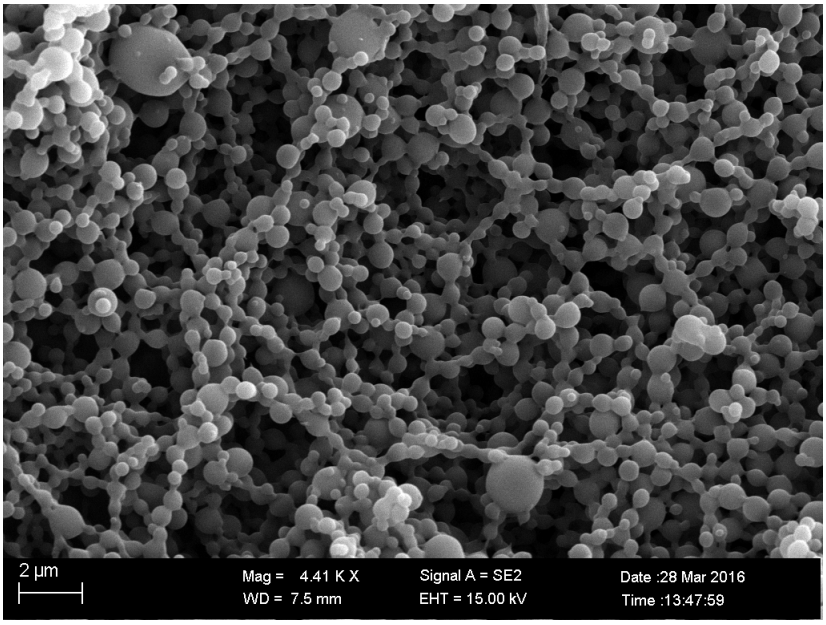


Figure B.5: Sample 21032016

Few conclusions of how the the different parameters affected the synthesis could be made since no particles could be observed. However the samples prepared dry for SEM-imaging at least showed results resembling of particles compared to the coral-like shapes obtained when the sample was first suspended. Dry sample preparation was therefore performed henceforth.

All samples displayed signs of melting. The only part in the synthesis process that involves generation of heat is the sonication, which is performed in an ice bath to avoid over-heating. To be able to rule this factor out two tests were performed with

very low sonication power and times. The two samples were performed with 50 W, 3x10 s and 10 W, 3x30 s sonication power and time. However the SEM images (not showed) looked like previous ones with hints of particles that had all melted together to from a network. It was therefore concluded that it was unlikely that the sonication power caused the melting. Further reduction of sonication power was anyways not an option because it would be insufficient for emulsification.

Another hypothesis was required to explain the lack of particles. The supervisor suggested to investigate the degree of hydrolyzation of the stabilizer, PVA. The degree of hydrolyzation, i.e. the amount of hydroxyl groups, affects the PVA-PVA interaction. Higher degrees of hydrolyzation increases the chances of hydrogen bonding between the hydroxyl groups of the surface-bonded PVA chains of different PLGA particles resulting in particle aggregation, see figure B.6. There is also an increase risk of self-bonding where the PVA-chain folds and binds itself or other free chains and forms flakes. According to Murakami et al. (1997) low degrees of hydrolyzation of PVA results in higher yields and more monodispersed PLGA nanoparticles while high degrees of hydrolyzation might cause particle aggregation. This is attributed to the decrease of hydroxyl groups that can hydrogen bond and the increase of acetyl groups which introduce a steric hindrance.

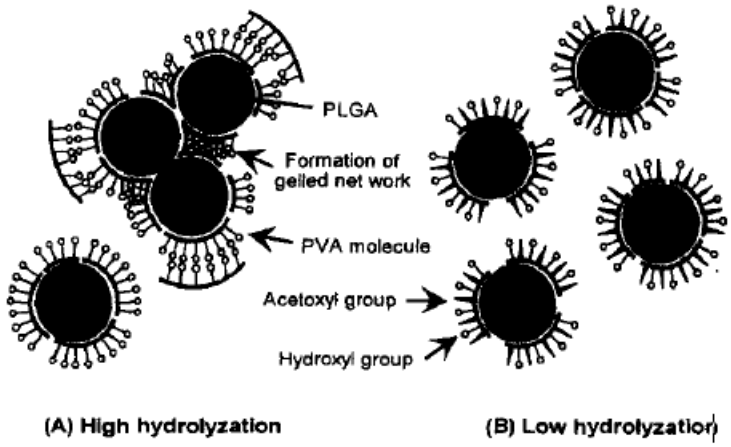


Figure B.6: Comparison between PVA with high and low degree of hydrolyzation. High hydrolyzation equals more hydroxyl groups which can hydrogen bond with each other and thus increasing the risk for particle aggregation.
Source: Murakami et al. (1997)

Prior tests had used 99.9% hydrolyzed PVA. A switch was made to PVA with 87-90% degree of hydrolyzation, having a molecular weight of 30-70 kDa instead of 89-98 kDa. Several particle syntheses were performed using different concentrations of 87-90% hydrolyzed PVA. In one test Vitamin E TPGS, a stabilizer recommended by McCall & Sirianni (2013), was tested. Even with the changes in stabilizer the SEM images, not showed here, continued to display a coral-like appearance.

The situation started to look grim. A lot of the project time had been used up without achieving any nanoparticles. Lab work was performed daily but the scientific value for my own sake was limited since only small variations were done to the protocol. The project started to feel repetitive and not very enriching. In an attempt to obtain a breakthrough a DLS was performed on the latest samples. Spherical particles are required for DLS results to be accurate, a property that had not yet been confirmed in the samples. In fact, judging from the SEM results there seemed to be no particles present at all. But there were some indications during the synthesis process that particles indeed had been synthesized, such as the emulsion was milky (a sign of a colloidal suspension) and the formation of a very fine powder after the lyophilization. A DLS measurement is fast and cheap to perform which made it worth the effort even though the prospect of reliable results was slim. The result can be seen in figure B.7.

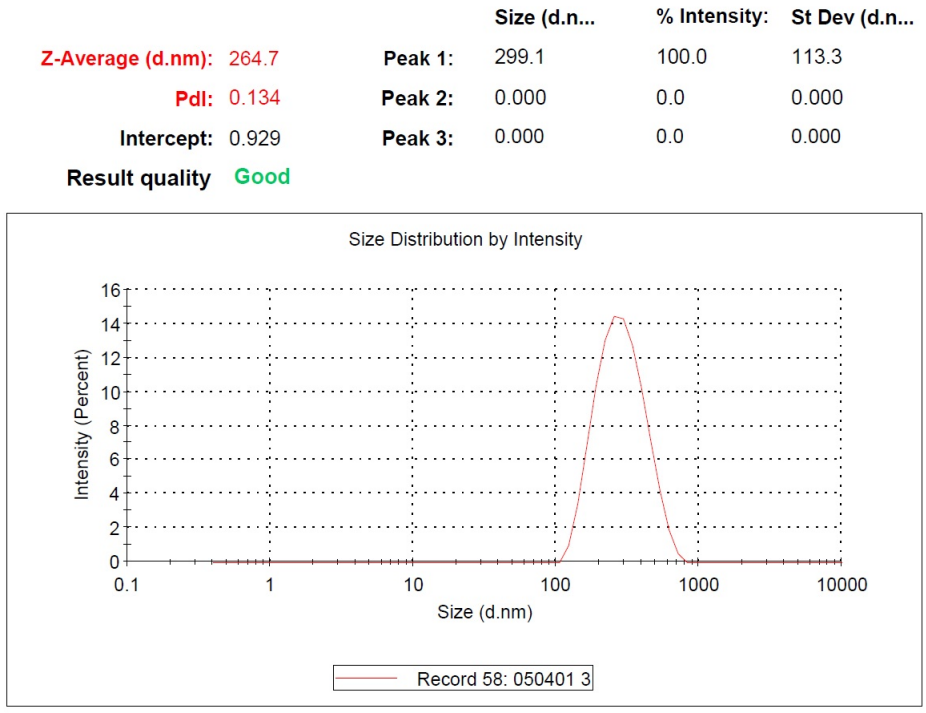
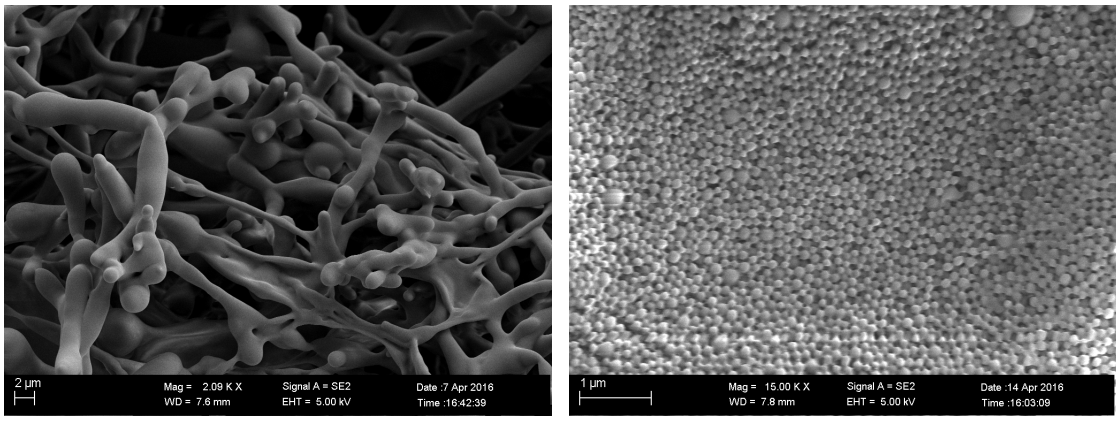


Figure B.7: DLS measurement of sample 05042016.

The result seen in B.7 was a big surprise! The graph behaved just as expected from spherical particles. The measurement was repeated several times and the result could be confirmed. The new working hypothesis was that spherical non-interconnected particles indeed were synthesized but something in the SEM-sample preparation made them agglomerate. The protocol was revised and SEM-sample preparation was researched both through literature and by consulting colleagues. A scientist from another research group recommended to use platinum coating for SEM imaging instead of gold since the gold sputtering might melt the heat sensitive PLGA. Upon further investigation it became clear that the colleague responsible for the promising results showed in B.1 also had used platinum coating. This was an unexpected development since several articles had used gold sputtering for their

PLGA nanoparticles. I have been unable to find any literature that support that gold sputtering involves more heat than platinum coating.

While waiting for the next available SEM time two particle batches were synthesized, sample 12042016a was a repeat of 05042016 in order to test the reproducibility of the protocol while 06042016a was made with decreased PVA concentration (from 5% to 1%). For the SEM imaging the samples, both new and old, were coated with platinum instead of gold and the change was striking. All results now clearly displayed spherical particles in the nanoscale, even the samples that had had a coral-like appearance on previous SEM images. A comparison between the same sample but with different metal coating can be seen in figure B.8. The results definitely concluded that the particles melted together because of the gold sputtering and henceforth platinum sputtering was used instead. It was a relief to finally find the solution and obtain spherical nanoparticles, but it was also a bit frustrating that nanoparticles probably had been successfully synthesized from the beginning. A lot of the project time had been spent troubleshooting the protocol and in the end the error was in a part in which we assumed we had no control over since it was handled by lab technicians.



(a) **(b)**

Figure B.8: (a)Sample 05042016, coated with gold. (b)Sample 05042016, coated with platinum.
Magnification varies

The SEM images all displayed nanoparticles but there were variations. The first sample synthesized with 87-90% hydrolyzed PVA, displayed in figure B.8 (b) showed very small particles (approximately 50 nm in diameter) which were monodispersed and quite spherical. They were interconnected, suggesting that even platinum coating evolves some heat. Sample 12042016a was a repeat of 05042016 and both samples looked identical, which indicates good reproducibility. Sample 06042016a had PVA concentration of 1% instead of 5% and the SEM result, seen in figure B.9 (a), showed slightly bigger and more polydispersed particles compared to sample 05042016 (with 5% PVA), seen in figure B.8 (b). This suggests that a high concentrations of stabi-

lizer facilitates the production of small monodispersed nanoparticles. When vitamin E TPGS was used as a stabilizer instead of PVA the particles became more deformed and less monodispersed, see sample 06042016b in figure B.9(b). Interestingly there seemed to be a distinct gap in size distribution where small particles surrounded bigger ones with no particles of intermediate size present. PVA was ruled to be a more efficient stabilizer to obtain small spherical nanoparticles and was used henceforth. Finally two samples using different solvents (ethyl acetate and dichloromethane) and different molecular weights of PLGA (20 kDa and 70-88 kDa respectively) were compared with no visual differences, see sample 23032016a (ethyl acetate) in B.9 (c) and sample 23032016b (dichloromethane) in B.9 (d) It would have been more appropriate test just one parameter at the time and keep the other static, e.i. do four samples instead of two, but since no difference could be seen it was assumed that solvent and molecular weight of polymer did not have a major impact on particle properties.

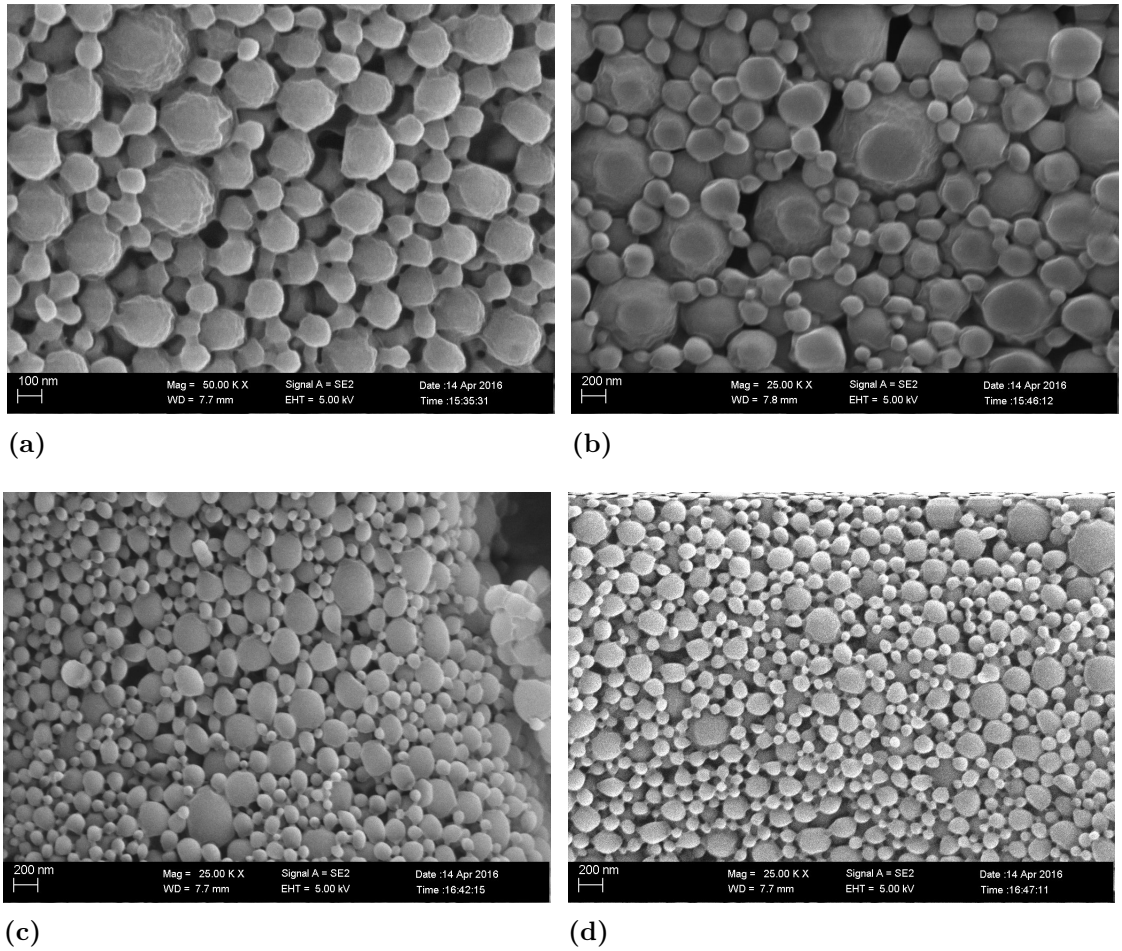


Figure B.9: (a) Sample 06042016a (1% PVA) (b)06042016b (Vitamin E TPGS) (c)23032016a (Ethyl acetate, PLGA: 20 kD) (d)23032016b (Dichloromethane PLGA:70-88 kD
Magnification 25k x

DLS measurements started to be carried out routinely since it had now been confirmed by the SEM results that the particles were more or less spherical. The size obtained from the DLS was always bigger than the one observed on the SEM images for different reasons; size determination by SEM-imaging is not a produced value but a qualitative estimation made by the scientist, a SEM image only shows a very small portion of the sample while DLS measures an average, DLS does not measure the actual size but the hydrodynamic diameter which is affected by shape of the particle and the properties of its surface as well as the ion concentration of the solvent, and finally $hydrodynamic\ diameter \geq diameter$ if the particle is a perfect hard sphere. Even if the two methods produced different results they always correlated, i.e. particles looking big on SEM images also produced a bigger size in the DLS results. It was therefore concluded that even if DLS was not exact it was a quick and simple method to obtain a good estimate of the particle size.

Now when it had been confirmed that spherical nanoparticles could be synthesized it was high time to start growing the cells. The cell experiments started with a period of training in order to get used to the cell lab, the working procedure and some of the planned tests. There was a big emphasis on sterilization routines and gentle handling of the cells in order to avoid infections. The cell line used was MC3T3, derived from mouse calvaria (skullcap). It is an osteoblastic cell line capable of differentiation into osteoblasts and is also considerable cheaper than hMSCs. The cells were thawed, grown and expanded. The medium consisted of DMEM HG (Dulbecco's Modified Eagle's Medium with High Glucose) together with 10% FBS (Fetal Bovine Serum) as a protein source and 1% antibiotics (penicillin and streptomycin) to avoid infections.

There were some problems with the thawing in the beginning. The cells did not seem to survive or were dormant. This could be explained by the age of the cells or in what condition they were in when they were frozen. Another perhaps more plausible explanation is my lack of experience. After a few attempts the cells were successfully thawed, however they did not grow. They appeared to be viable when studied through a microscope but had only achieved approximately 60% confluency after 16 days of growth. They were then discarded and no more MC3T3 cells were thawed in order to save both time and resources. Luckily my more experienced lab partner Simon Myrbäck had more success with his cells and I had the opportunity to obtain valuable experience by aiding him in his work. The training involved thawing, expanding and plating of both MC3T3s and hMSCs.

Particle syntheses were performed simultaneously with the cell training. The aim had shifted from investigating the synthesis parameters to obtaining batches of PLGA nanoparticles with significant size differences. Previous experiments had concluded that stabilizer concentration and sonication power had high impact on the result. Sample 27042016a was made with high stabilizer concentration (5% PVA) and high sonication power (700 W) in order to obtain small particles while 27042016b had lower PVA concentration (0.5%) and sonication power (200 W) to achieve bigger particles. The SEM results confirmed the expectations but also presented a few possible obstacles. As intended the particles in sample 27042016a were

very small, approximately only 50 nm in diameter, and monodispersed. The particles of sample 27042016b were significantly bigger, ranging from 200 nm to almost a micron, and quite polydispersed. This was expected since, simply put, the sonication power governs the nature of the emulsion which forms the particles while the stabilizer concentration aids in maintaining it. However the smaller particles were interconnected to a greater extent and seem to more heat sensitive compared to bigger particles. It was assumed that the deformation was caused by the platinum sputtering. The risk of particle melting during cell tests at room temperature was deemed very small. The DLS results further supported spherical non-interconnected particles. The average hydrodynamic diameter of sample 27042016a was 250 nm while sample 27042016b was approximately twice as big.

While the samples looked promising it became evident that insufficient yields might pose a problem. This seemed to be especially prominent for small particles. 38% of the PLGA used in sample 27042016b was transformed into particles and the same number for sample 27042016a was only 5%. Given that 200 mg of PLGA is used for each sample the resulting amount of particles in sample 27042017a became only 5 mg which is not enough for both characterization and cell tests. No material losses had been observed during the synthesis which led to the working hypothesis the particle collection step could be improved. The centrifugation speed was therefore increased in the following synthesis in order to collect smaller particles. Another aim was to increase the size of the bigger particles to ensure significant size difference. This was performed by reducing the sonication power from 200 W to 50 W. The sonication time was increased from 3x10s to 3x30s to ensure complete emulsification.

Unfortunately the vial containing the sample with increased centrifugation speed broke during the particle collection step and the sample was lost. Even so some knowledge could be salvaged from the synthesis. It was very difficult to resuspend the pellet due to the high centrifugation speed. It was instead decided to try to increase the yield by slightly increasing the size of the particles. The other sample, 04052016b, obtained a satisfying polymer yield of 56%.

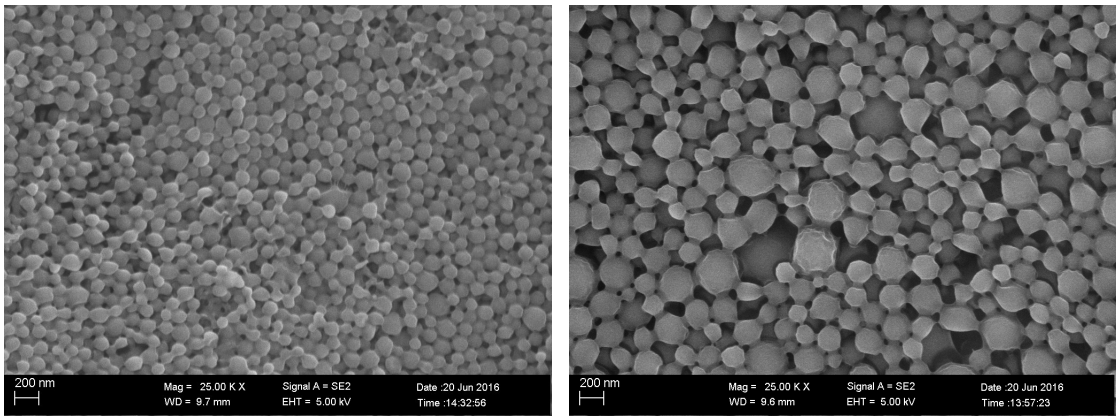
Since there seemed to be a correlation between particle size and polymer yield sample 08052016a was synthesized with the aim to increase the size by slightly decreasing sonication power from 700 W to 400 W. The final yield was still low, 9.7%, but sufficient for cell tests and characterization. It was assumed that 08052016a was significantly smaller than 04052016b. This could however not be verified yet because no characterization method was available at the time due to renovations. Even so it was decided to do a cell viable test with CCK-8 where hMSCs were subjected to different particle concentrations of 04052016b and 08052016a. The viability test was carried out for three main reasons, to test the cytotoxicity of the particles, to investigate if particle size affect the viability and as a training for upcoming experiments. The results were difficult to interpret due to big variances and were probably not representative. However no trend could be seen that implied that the PLGA nanoparticles were cytotoxic nor that neither particle size or concentration had significant impact on cell viability.

Later characterization of the particles by DLS produced unreliable results for sample 04052016b, an indication that the synthesis was unsuccessful and the particles might not be spherical. The results of sample 08052016a looked promising however and showed a particle size of 240 nm. This was surprising because the size was expected to be bigger than 27042016a (which had a hydrodynamic diameter of 250 nm) due to the decreased sonication power.

New attempts were made to synthesis particles of different sizes. The idea was to keep high concentrations of PVA for emulsion stability and monodispersity and instead vary the sonication power. Literature and former experiments had strongly suggested that sonication power had a negative correlation to particle size. Therefore the sonication power of samples 26052016a and 26052016b was set to 400 W and 50 W respectively. The yields were still small, 4.2% for sample 26052016a and 13.8% for 26052016b. This further supports the hypothesis that smaller particles results in lower yields with the current particle collection settings. According to DLS measurements the hydrodynamic diameter of the samples was 200 nm for 26052016a and 220 nm for 26052016b. The very small size difference was interesting for two reasons, the first being it suggested that the assumed impact of sonication power on particle size needed to be reevaluated. Secondly there seemed to be a narrow size interval where the centrifugation step starts to be effective in collecting the particles. Just an increase of 20 nm in particle diameter led to three times higher yields. Unfortunately no SEM time was available at the time for characterization.

The ability to synthesize small particles had been confirmed on several occasion. The present challenge was, a bit unexpectedly, to produce big particles. The aim was to produce monodispersed particles big enough to obtain a significant size difference, but preferably not exceeding 1 micron. Big particles also seemed to be synthesized more efficiently and a a rough estimate was that particle sizes of $\geq$300 nm would produce sufficient yields. In the final particle synthesis both sonication power and PVA concentration were varied to obtain as big size difference as possible. Sample 09062016a was meant to consist of small particles and the sonication power used was set to 10 times higher compared to sample 09062016b, 500 W and 50 W respectively. The PVA concentration of 090620162b was reduced from 5% to 1%, a value which was assumed to be low enough to have an impact but also sufficient to obtain monodispersity.

SEM images of the samples showed that the particles were monodispersed and slightly interconnected, see figure B.10. The average particle sizes were approximately 50 and 200 nm for 09062016a and 09062016b respectively. Both samples were quickly deformed by the electron beam even though only 5 kV was used. The DLS results looked promising for both samples and showed a hydrodynamic diameter of 250 nm for 09062016a and 350 nm for 09062016b. This was however not a sufficient difference since the size distribution curve had a big overlap, see figure B.11. There was a big difference in yields between the two samples, 6.1% for 09062016a and 46% for 09062016b.



(a) **(b)**

Figure B.10: (a)09062016a (b)09062016b
Magnification: 25k x

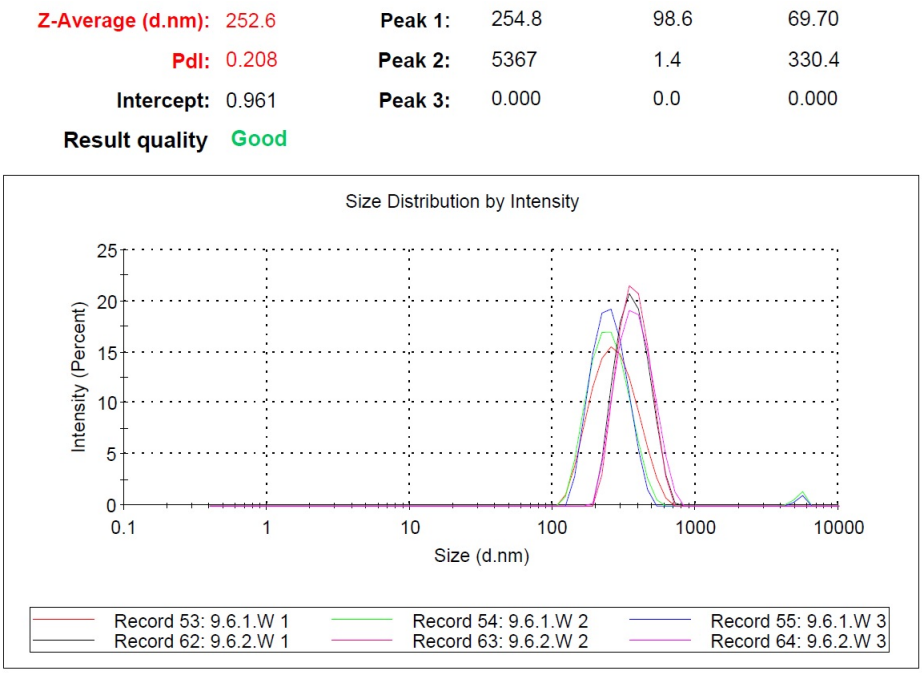


Figure B.11: DLS measurements of sample 09062016a and 09062016b in the same graphs displays a big overlap.

The main aim of the project was at this time changed from investigating the effect of PLGA nanoparticles of different sizes on hMSCs to just using one size but in different concentrations. The yield of sample 09062016a was too low to be used in the cell tests and even if it had been sufficient the size difference should have been too low to have any meaningful impact. The particles of sample 09062016b were spherical, monodispersed, of satisfying size and available in large amounts and was therefore used in all following experiments.

Sample 090620016b, henceforth referred to as the nanoparticles, was sterilized with γ-irradiation (20 kGy) and introduced to the hMSCs via the medium. The medium was changed after 24 h to new medium without nanoparticles and osteogenic induction was commenced. A CCK-8 viability test was performed to test the cytotoxicity of the particles and if the viability was concentration dependent. The osteogenic activity was examined with ALP-staining and Alizarin Red S staining. ALP-stains for alkaline phosphatase which is an enzyme expressed at the early stages of osteogenic differentiation. Alizarin Red S stains extracellular calcium which is deposited by osteoblasts. These were the final experiments and they are described in detail in the chapter 3 *Method* and in chapter 4 *Results*.

C

Appendix 3

C.1 FDA approved PLA/PLGA products

Table 1. PLA/PLGA-based drug products that are available in the U.S.

Drug Product	Active Ingredient	Dosage Form, Route of Administration	Strength,	Approval Date(s), Indication(s)	Characteristics of PLA/PLGA (described in product labeling)
Vivitrol	Naltrexone	Microsphere, Intramuscular	380 mg every 4-weeks	1984, Indicated for the treatment of alcohol dependence	PLGA, L/G: 75/25
Zoladex	Goserelin acetate	Implant, Subcutaneous	3.6 mg or 10.8 mg every 28 days	1989 (3.6 mg), 1996 (10.8 mg), Indicated for use in combination with flutamide for the management of locally confined Stage T2b-T4 carcinoma of the prostate	PLGA (13.3-14.3 mg/dose) No characterization information
Lupron Depot	Leuprolide acetate	Microsphere, Intramuscular	7.5 mg, every month 22.5 mg, every 3-months 30 mg, every 4-months 45 mg, every 6-months	1989, Indicated for palliative treatment of advanced prostatic cancer	7.5 mg: PLGA (66.2 mg/dose) 22.5 mg: PLA (198.6 mg/dose) 30 mg: PLA (264.8 mg/dose) 45 mg: PLA (169.9 mg/dose) No characterization information
Lupron Depot-PED	Leuprolide acetate	Microsphere, Intramuscular	7.5 mg, 11.25 mg, or 15 mg every month 11.25 mg or 30 mg every 3-months	1993, Indicated for the treatment of children with central precocious puberty (CPP)	7.5 mg, 11.25 mg, and 15 mg: PLGA (66.2/99.3/132.4 mg/dose) 11.25 mg and 30 mg: PLA (99.3/264.8 mg/dose) No characterization information
Lupron	Leuprolide acetate	Microsphere, Intramuscular	3.75 mg, every month	1995, Indicated for management of endometriosis	PLGA (33.1 mg/dose) No characterization information
Sandostatin LAR	Octreotide	Microsphere, Subcutaneous	10 mg, 20 mg, or 30 mg every 4-weeks	1998, Indicated for acromegaly, severe diarrhea/flushing episodes associated with metastatic carcinoid tumors, profuse watery diarrhea associated with VIP-secreting tumors	10 mg, 20 mg, and 30 mg: glucose star polymer, PLGA (188.8/377.6/566.4 mg/dose)
Atridox	Doxycycline hyclate	In situ forming gel Periodontal	50 mg	1998, Indicated for the treatment of chronic adult periodontitis for a gain in clinical attachment, reduction in probing depth, and reduction in bleeding on probing	PLA (36.7%/dose) No characterization data
Trelstar	Triptorelin pamoate	Microsphere, Intramuscular	3.75 mg every 4-weeks 11.25 mg every 12-weeks 22.5 mg every 24-weeks	2000 (3.75 mg), 2001 (11.25 mg), 2010 (22.5 mg), Indicated for the palliative treatment of advanced prostate cancer	3.75 mg, 11.25 mg, and 22.5 mg: PLGA (136/118/182 mg/dose) No characterization information
Arestin	Minocycline HCl	Microsphere, Periodontal	1 mg, variable dosing frequency	2001, Indicated as an adjunct to scaling and root planning procedures in patients with adult periodontitis	PLGA No characterization information
Eligard	Leuprolide acetate	In situ forming gel, Subcutaneous	7.5 mg every month 22.5 mg every 3-months 30 mg every 4-months 45 mg every 6-months	2002 (7.5 mg and 22.5 mg), 2003 (30 mg), and 2004 (45 mg), Indicated for the palliative treatment of advanced prostate cancer	7.5 mg: PLGA (82.5 mg/dose), carboxyl endgroups, L/G: 50/50 22.5 mg and 30 mg: PLGA (158.6/211.5 mg/dose), copolymer with hexanediol, L/G: 75/25 45 mg: PLGA (165 mg/dose), copolymer with hexanediol, L/G: 85/15
Risperdal Consta	Risperidone	Microsphere, Intramuscular	12.5 mg, 25 mg, 37.5 mg, or 50 mg every 2-weeks	2003, Indicated for the treatment of schizophrenia and bipolar I disorder	12.5 mg, 25 mg, 37,5 mg, and 50 mg: PLGA, L/G: 72/25
Ozurdex	Dexamethasone	Microsphere, Subcutaneous	0.7 mg, variable dosing frequency	2009, Indicated for the treatment of macular edema, non-infectious uveitis, and diabetic macular edema	PLGA No characterization information
Bydureon	Exenatide	Microsphere; Tablet	2 mg, every 7-days	2012, Indicated as an adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes	PLGA (37.2 mg/dose), L/G: 50/50
Lupaneta Pack	Leuprolide acetate; Norethindrone acetate	Intramuscular; Oral	3.75 mg every month; 5 mg daily	2012, Indicated for initial management of the painful symptoms of endometriosis and management of recurrence of symptoms	DL-lactic and glycolic acids copolymer (33.1 mg), no characterization data
Signifor LAR	Pasireotide pamoate	Microsphere, Intramuscular	20 mg, 40 mg, or 60 mg every 28-days	2014, Indicated for the treatment of patients with acromegaly	20 mg, 40 mg, and 60 mg: a mixture of two PLGAs per dose PLGA I (26.29/52.58/78.87 mg/dose), L/G: 50-60/40-50 PLGA II (26.29/52.58/78.87 mg/dose), L/G: 50/50

Figure C.1: PLA/PLGA-based drug products that are available in the U.S. market in June, 2016.
Source American Pharmaceutical Review (2016)