SYNTHESIS OF MESOPOPROUS SILICA NANOPARTICLES USING DROPLET MICROFLUIDICS

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ABSTRACT

Introduction: Nanoparticles are small particles within nanoscale levels. Their size goes up to few hundred nanometers, although some sources state that nanoparticles are up to 100nm in diameter. Structure and function can vary between different nanoparticle models, each depending on physical properties of particles as well as way of their production. Each particle represents one functional unit. One of the most used type of nanoparticle is mesoporous silica nanoparticle. This is round shaped nanoparticle made of mesoporous silica, which makes it widespread when it comes to the drug loading. Despite the thing that nanomedicine sounds perfect in theory and brings enormous potential into targeted drug delivery, in real life it is hard to predict its behavior in vitro, and especially in vivo.

Objectives: One way to enhance synthesis of MSNs and improve its efficiency is use of microfluidic chips and techniques. Microfluidic chip brings opportunity to manipulate different fluid flow in order to synthesize nanoparticles inside a picoliter volume droplets. The first objective is to optimize the microfluidic system in order to create stable droplets in order to synthesize MSNs inside a droplet. Second objective was to wash out the sample and measure the particles in order to check their size. Third objective was to image the particles with TEM (Transmission Electron Microscope) to see if their shape and size are suitable for drug loading, coating and similar manipulations, as well as establishing the protocol for the full process.

Methods: Optimization is conducted by adjusting different flow rates and concentration of CTAB, TEOS, SPAN65 which are surfactants, precipitates and solvents. The main goal of optimization is to create stable fluid flow and stable droplets. Optimization process is monitored in real time with high speed microscope camera. Fluid flows of each substance were adjusted with fluid flow pumps. The main goal is to create a stable flow thus having a stable droplets in a sample. After the formation and collection of stable droplets, the sample is centrifuged and washed with ammonium hydroxide and ethanol solution for three times. After washing sample should be taken to Zetasizer, in order to measure particle size. If the sample is within certain nanometer range, it will be stored and imaged with Transmission electron microscope. Obtained images will be prone to image analysis with imageJ, from which data analysis will be obtained as well.

Keywords: Mesoporous Silica Nanoparticle (MSN), Transmission Electron Microscope (TEM), Cetyl Trimethyl Ammonium Bromide (CTAB), Tetra Orto Silicate (TEOS)

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1. LITERATURE REVIEW

3.2 Nanoparticles

Nanoparticles are the core component of the nanotechnology field, which is rapidly emerging field, tightly correlated with drug delivery (Kumar et al., 2018).

Nanoparticles represent particles that have size within certain nanoscale, usually between 1 and 100 nanometres in their diameter. They can form many shapes such as round, rod or prism shaped, and they are all unique for their properties (Vollath et al., 2018). Since the size of the nanoparticles is up to 100nm, it is clear that they cannot be seen within certain light wavelengths, since their size is smaller than light wavelengths which usually ranges between 350-700nm and is known as visible light. This is the reason why it is needed to use electron microscopy in order to image or see nanoparticles (Khan et al., 2019). Even that nanoparticles are synthesized for their purpose, some of the nanoparticles can occur naturally. (Plane, 2012)

As majority of nanoparticles is synthesized, that has been done in a way that they form certain shape, which also dictates its function, especially in the field of drug delivery and nanotechnology. They can form an excessive number of shapes, ranging from simple to more complex ones. This shape can be simple as spherical or nonspherical shape such as rod, star shape, all the way up to forming chains of similar shaped nanoparticles (Guo et al., 2014). Properties of each nanoparticle dictate their function, and their behaviour. These properties encompass size of the particle, its shape, charge, thermal properties and density (Khan et al., 2019). NPs can be divided in many groups according to their type or properties. Classification of nanoparticles can be different due to chemical properties, size, morphology and physical properties (Crucho and Barros, 2017). According to their synthesis way and chemical properties they can be carbon based, silica nanoparticles, ceramic nanoparticles, metal core nanoparticles, semiconductor nanoparticles, as well as polymeric and lipid based (Guo et al., 2014).

Different nanoparticles have different uses in scientific researches, pharmacy and also consumer industry.

3.3 Mesoporous Silica Nanoparticles

Nanoparticles can be synthesized and developed from many materials and with different methods, but one kind of nanoparticles especially stands out when it comes to numerous studies, and it is mesoporous silica nanoparticles (Mohanraj and Chen, 2006). What makes MSNs to stand out is their unique properties which make them great potential for drug delivery. Some of these properties are their physical properties which include high surface area, unique structure, ease of modification of the physical properties and low toxicity. All of these properties are adjustable and prone to change, and that is what makes these nanoparticles special and suitable for drug delivery. All of these bring opportunity to increase drug absorption, drug loading and release (Wang et al., 2015). The fact that is very specific for MSNs is that pore diameter can range between 2 and 50 nanometres. This wide range within nanoscale brings many different opportunities for usage of these nanoparticles. The size of the whole particle can be changed and tuned with ease, which also affects the pore size and number of pores. One fact that makes MSNs unique is that they have two surfaces, inner and outer, which means doubling the function of the particles. (Pednekar et al., 2017)

Since one of the main uses of MSNs is drug delivery, it is very important to consider the effect of the nanoparticle itself to the body. This means if the particle is cytotoxic and biodegradable and if so, to what extent. When it comes to mesoporous silica nanoparticles, they have high biodegradability and low to none cytotoxicity, depending on their size. The hydrolytic degradability of these particles is almost 100%, but time of it may vary depending on a size of particle, as well as the surroundings of a particle, which is different for different organ or cell types (Croissant et al., 2017)

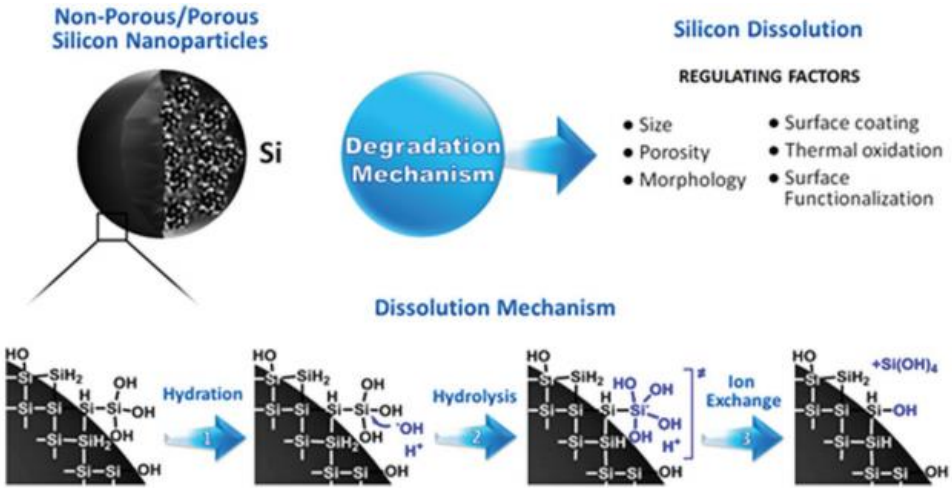


Figure 1. Representation of degradation mechanism and regulating dissolution factors of MSNs (Croissant et al., 2017)

3.3.1 Synthesis of MSNs

As mesoporous silica nanoparticles have vital role in drug delivery field due to their properties, it is crucial to be able to change these properties accordingly. This is done with the synthesis of the MSNs, and different settings and components used in synthesis dictate the properties of MSNs. Since they load specific drugs, it is crucial that drug affects just the targeted area, and this depends on the particle properties (Vallet-Regí et al., 2017). There are different synthesis methods, but one can be considered most common and best established, and this method is known as sol-gel method. This method is consisted of creating silica carriers which synthesizes from tetraethyl orthosilicate known as TEOS, and cetyltrimethylammonium bromide (CTAB) as template and pore generator at the same time. Properties can be altered by changing the ratio of water and TEOS, as well as CTAB concentration. Since CTAB is surfactant, its amount highly affects surface area and the number of pores. Cetyltrimethylammonium bromide is used as a template for silica precursors, and it is in form of micelles, so its concentration affects the number and size of surfactant micelles, which finally affects the size of particles and their porosity. (Vazquez et al., 2017)

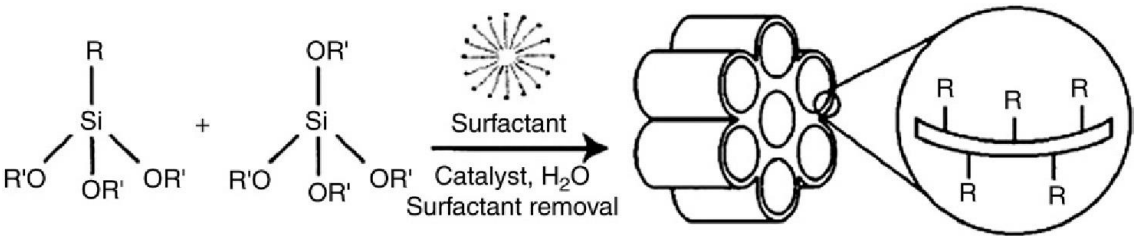


Figure 2. Illustration of MSN production and its synthesis within the sol gel method (Pednekar et al., 2017)

Particles produced with sol-gel method are measured and characterised by dynamic light scattering and zeta potential count. Synthesis properties affect the size and shape of the particle, as well as their morphology. Although sol-gel method is effective, it is time consuming, and not fully efficient, since all the particles are synthesized within bulk and moderate number of them is not synthesized properly and unusable (Vazquez et al., 2017); (Alemán et al., 2007).

Depending on a synthesis properties, different functions and tasks can be assigned to the MSNs, leading them to have extremely high loading capacity, as well as carry different drugs, which makes them superior to the orthodox drug delivery systems, due to their flexibility, versatility and reliability. (Tang et al., 2012)

3.3.2 CTAB

Cetyl trimethylammonium bromide or CTAB is one of the main components needed in MSN synthesis. It is surfactant that forms micelles that behave as pattern for mesoporous silica nanoparticles (He et al., 2018). Despite the fact that it is vastly useful during the synthesis, CTAB is actually highly cytotoxic which is quite contrary to the purpose of nanoparticles. In order to get the full potential of MSNs, CTAB needs to be washed out, especially if nanoparticle is going to be applied in vivo (Wang et al., 2013). CTAB is very important in nanoparticle synthesis, because it is reactive with water, where it forms micelles. Its main purpose is forming the template and maintaining the shape of the particle until it completely synthesises. CTAB needs to be washed out of the sample due to its cytotoxicity, but excessive washing can also affect the product itself since a lot of product can be lost after CTAB is gone to maintain the desired shape. (Becker et al., 2010)

3.3.3 Application of MSNs

Mesoporous silica nanoparticles present one of the major components in drug delivery field and pharmacokinetics, and they have found place in different fields such as drug delivery, protein delivery, cancer therapy, gene delivery and different responsive releases (Pednekar et al., 2017). When it comes to the cancer therapy, main problem is that cancer drugs are usually instable, have poor solubility and very often don reach targeted cells as effective as they should. This is due to reactivity of the drug with its surroundings, which makes it less effective by the time it reaches targeted area. These problems can be compensated with loading the drug inside a stable mesoporous silica nanoparticle. By doing this, drug gets physical protection on the way to the cancer cells, which minimizes reaction and release of the drug before time, lowering drug degradation, thus increasing the concentration of the drug that reaches targeted area as well as drug uptake of cancer cells (Mellaerts et al., 2008)

Similar to the cancer therapy concept, delivery of certain proteins can be used in therapeutic purposes. Protein as a molecule has a large weight and tertiary structure which makes it highly reactive. Protein molecules can be used in medical application as a targeted protein that reacts with targeted area (Senapati, 2018). As stated, protein is highly reactive, meaning it changes its structure and function quite easily, leading it to complete loss of its biological activity. This represents the biggest problem in drug delivery, which can be solved by loading protein to the MSN. By doing this, protein can easily be preserved and protected on its way to targeted area by porous structure that remains stable all the way up to targeted cells, thus increasing efficiency of the drug. (Sood and Panchagnula, 2001)

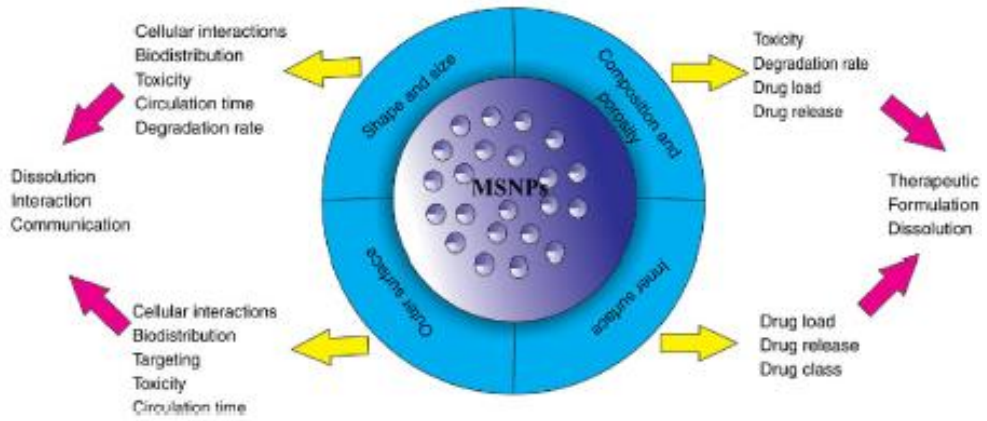


Figure 3. MSN characteristics and their effect on particle behaviour (Pednekar et al., 2017)

One of the promising but hard to execute methods in targeted drug delivery is using genes in therapeutic purposes. Therapeutic genes can be in form of plasmid DNA, short RNA chains, and chain of just few nucleotides, all of them highly potent in repairing broken cell mechanisms and cancer therapy (Tang et al., 2012). Since charge of these molecules is negative, their half-life is also short due to their biological instability. Even they have high potential in targeted therapy, it is nearly impossible to transfer them to desired cell without them reacting with other cells on the way. One way to compensate this is using viral carrier, which is not considered safe as it can have undesired counter effects on healthy cells (Slowing et al., 2008). This problem can be solved by modifying MSN pore size and loading genes into the nanoparticle. MSN behaves as nonviral carrier, and when combined with certain polymers can create positively charged surface, which creates strong bond with negative charged gene, minimising chances of gene interacting with other cells before it reaches targeted cell (Gao et al., 2009); (Garg et al., 2011) .With smaller modification to the MSNs, such as increasing pore diameters in nanometres, it is possible to load whole plasmids into mesoporous silica nanoparticle, leaving the plasmid protected and maintaining its form all the way until interacting with targeted cell. (Li et al., 2011)

The way of making MSNs fully potent in a way that they do not interact with any of the cells on the way then just the targeted cells, thus maximising drug absorption in the desired area, requires specific approach. This approach is called triggered release and it is stimulated by factors already presented in the organism, called internal stimulus, or external stimulus represented in predetermined pathway. Triggered release is a concept of major significance when it comes to targeted drug delivery, and it allows drug, genes

or proteins to be released to exact location, and in time and dosage controlled fashion. As far as external factors go, they can be used to enhance the drug delivery in manner of time, location and dosage of released drug. Most used external triggers are temperature, magnetic field, light, pH, temperature and use of enzymes. (Sun, 2012)

In order for MSN to react with certain pH, modifications of particle need to be done, such as adding components that detach the cargo when in certain pH environment, for example acid environment around tumor cells (Nguyen et al., 2006). One of many examples is also adding shell on MSN which can open and close, depending on surrounding pH, meaning that it releases cargo from pores in desired environment, with corresponding pH value (Cauda et al., 2010)

One of the simple but fascinating uses of MSNs is the drug release that is triggered by change in temperature. This is very useful in cancer therapy, since tumor cells have higher temperature than healthy cells. By this, it is possible to keep drug unreleased in normal circulation, thus delivering drug with full dose and full potential to cancer cells only (Fu et al., 2007). For example, it is possible to modify MSN by adding thermo-sensitive polymer that covers the pores of mesoporous silica nanoparticles, which changes its conformation in environment with higher temperature, thus leaving pores open and releasing the drug to targeted area. (Pelton, 2000)

Convenient and remote controlled drug delivery to desired site can be executed with light irradiation. Light is radiation, represented by electromagnetic wavelength (Sliney et al., 1976). By exposing targeted area to certain light wavelengths, it is possible to induce drug release and drug uptake to from MSNs to targeted cells. In order to get this effect, it is necessary to make slight modification to MSNs by adding linkers that have photochemical response. These linkers are built in nanoparticle surface and cover the pores, but after the stimulus by light, they change their structure and leave pores open, leading to release of the drug. (Ferris et al., 2009)

3.3.4 Biocompatibility

The rapid evolution in development of nanoparticles in general, but especially mesoporous silica nanoparticles has moved them to one of the most potent solutions when it comes to targeted drug delivery and nanomedicine fields. Having so many potential modifications and variable properties make it theoretically solution for every

targeted drug delivery problem, but in practice, there are still many issues, and one of them is question of MSNs biocompatibility and cytotoxicity. (Chithrani et al., 2006)

Regarding of cytotoxicity, it is considered low, or there is no remarkable cytotoxicity, depending on the dosage. Some of the smaller particles may affect some cells, such as human dendritic cells and their viability, as well as erythrocytes. Some in vitro experiments show that cytotoxicity is directly proportional to size and morphology of MSN. MSNs do have cytotoxicity properties which are highly dependant on their size, morphology and targeted area. Some research did show small amounts of cytotoxicity in some experiments, such as aforementioned to red blood cells and dendritic cells, but also in some researches that observed long term studies, MSNs would accumulate in liver for few months but showing zero signs of cytotoxicity. Most of these results are taken in vitro, and cannot be observed as final as they require more investigation and research (Sun, 2012). It was established that cellular uptake does depend on the size of particle, as well as that distribution of nanoparticles follow different patterns for different size of particle in nanometres (He et al., 2011). There are two most important parameters to consider regarding biocompatibility and those are size and surface. Since MSNs are prone to many variations, and allow many properties to be modified, it does affect there cytotoxicity, which in general, can be considered low for all MSNs (He et al., 2010).

3.4 Microfluidics

Microfluidics represents one of the most emerging areas correlated with pharmacokinetics and drug delivery, and it is combination of different fields such as physics, chemistry, biology and biomedicine. These systems also use constant use of optics and imaging devices, making unique fusion often referred to as optofluidics. (Wu et al., 2012)

Microfluidics offer the new concept what is called lab on a chip, which means that it is possible to take the big experiments on a microscale. This idea of lab on a chip can be considered revolutionising, because it brings opportunity to use smaller volumes of reagents and chemicals used, conduct the experiments on smaller scale, making it less expensive and more affordable worldwide. Smaller scales bring shorter time of conducting the research which can be crucial especially in field of targeted drug delivery (Figeys and Pinto). What makes microfluidics so interesting and outstanding is

the physics behind microscale. Since everything is happening on much smaller scale, it is possible to use the law of the physics in a way that is unimaginable on a macroscale. This brings so many opportunities, and also less cost, which is quite important today (Beebe et al., 2014).

Starting point of every microfluidic research is construction of microchip, a place where whole experiment will be done, in a microscale. There are commercially available microchips, but it is possible to build and design microchip for experiment, by understanding the physics on a microscale (Brody et al., 1996). In order to successfully design a chip, there are few effects that need to be considered beforehand in order to get most comprehensive idea of what lab on a chip should look like, and how it should behave. These effects are surface to volume ratio, tension, fluidic resistance, diffusion and laminar flow. These are all physic concepts that are crucial in order to use chip in fields of biology, biomedicine and drug delivery (Beebe et al., 2014).

Laminar flow represents condition where fluid stream is not considered to be a random. Since channels in microchip are small sized, flow can be considered almost always laminar. When it comes to the laminar flow, in order to mix two or more fluid flows, diffusion is required. One of the features of laminar flow is the possibility to create flows that maintain form, which leads to creation of droplets inside solution, and it is possible to affect their size and properties. All of this is done by using diffusion between two laminar fluid flows (Glass et al., 2008).

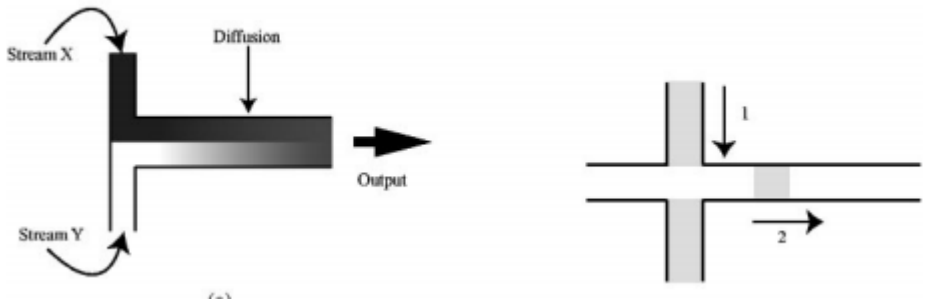


Figure 4. On the picture above, example of diffusion and fluid flows within the chip are shown. On the left side it is illustration of two different flows that will not mix except by diffusion, while on the right side it is shown how two different directions of the flow move a part of one flow through the channel. The example on a right side can be used in creating droplets (Beebe et al., 2014)

By using all of these physics concepts, it is possible to build a lab on a chip which will bring high impact to the field, by allowing manipulation of fluid flows on a microscale levels. (Papautsky and Bhagat, 2013)

Microfluidic system brings high operation rates on the table, which gives it significance especially when it comes to bioparticle application in clinical diagnostics where time is of the essence (Di Carlo, 2009). Optical imaging is also tightly correlated to microfluidic systems. It is essential to visualisation of lab on chip, as well as providing constant monitoring which is of major significance when it comes to the microscale experiments. Combination of optical imaging and microfluidic devices provides low cost and portable solution (Wu et al., 2012).

3.5 HLB or Hydrophilic-lipophilic balance

Hydrophilic-Lipophilic Balance is a scale that represents the measure of hydrophilic and hydrophobic of certain solution or element, most commonly surfactant, more precisely non-ionic surfactants. The reason why is this balance shown in a scale form is to better visualise the difference and balance between properties. Since surfactants are used to stabilise the emulsion, it is hard to predict how exactly they will affect the emulsion without knowing their hydrophobicity/hydrophilicity properties. HLB scale ranges from values of 0 as a lowest one on HLB scale, to value of 18 as highest possible value of Hydrophilic-Lipophilic Balance (Ohshima et al., 2016). HLB value represents parameter used to predict and asses the size of the core micelles, which also helps understand the charge of the bulk solution. Knowing this, it is easy to assume that surfactant can stabilize the emulsion and its charge, as well as affect particle charge in bulk solution, all depending on surfactants HLB value. (Gacek and Berg, 2015)

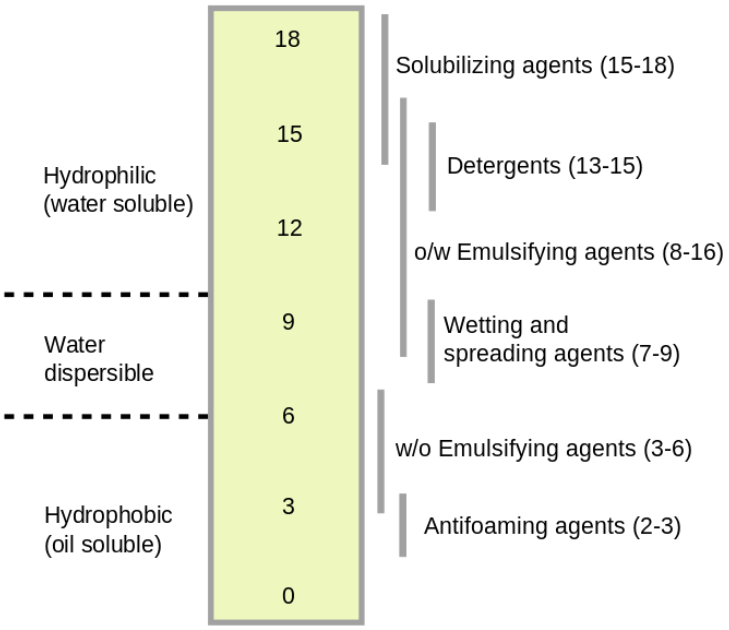


Figure 5. Sample of most common HLB scale as well as certain agents and surfactants with their values on the scale

HLB can be calculated by different simple math formulas, depending on a surfactant properties. It should be noted that HLB is affected by temperature, as well as presence of other agents that change pH value of the solution (Schott, 1995) One of the most recent and convenient ways to easily determine the HLB balance of surfactants by using inversion temperature deviation. This method is reliable and fast, which makes it perfect for double checking the HLB balance of surfactant of the interest. (Nollet et al., 2019)

2. HYPOTHESIS AND AIMS

Nanoparticles are greatly explored and their synthesis is well established. Theoretically nanoparticles are one of the best things that happened to drug delivery research, because they can provide many different usages and solutions. They can be synthesized in few different ways, using already established protocols. These protocols are convenient and bring solid results. But as every method, it has its setbacks and can be majorly improved. Usual nanoparticle synthesis protocols require a lot of time, and bring synthesis in a bulk. This means that big number of nanoparticle is created within suspension, and may affect the final product. Having extremely big number of particles within small space brings issues such as them interacting, sticking together, thus making them useless. And with high number of nanoparticle within suspension, it is high chance of them interacting and not being suitable for final product.

One way of improving these issues is introducing microfluidic systems in the mix. It takes similar principle as a bulk method, but with major improvements in the execution. By moving synthesis inside a microfluidic chip, it is possible to create a droplets that will behave as a microreactor, and with each droplet produced it is possible to produce nanoparticles within. That is exactly the aim of this research, introducing new method to synthesize nanoparticle, which in theory brings even more solutions and opportunities for both creating the nanoparticles, and using them, especially in field of drug delivery. By using microfluidics, it is possible to affect the speed and size of droplet creation, thus impacting synthesis of nanoparticle directly, by affecting their time to form, as well as size and physical properties. By creating a template for particle as well as all of its compound around it, all within microfluid chip, it is possible to create numerous droplets with nanoparticles inside. This would lead to easier creation of particles with different properties, as well as shortening the time for them to form by significant amount, and the biggest advantage would be their distribution within a droplet as a microreactor, instead of having millions of them inside one suspension.

Success in creating nanoparticles this way would also open the opportunity to load particles immediately with a protein or a drug for example, and all of that during the process of their synthesis, which could prove to be vastly significant.

Main aim of the research is to introduce a novel method, a new way of synthesizing nanoparticles more effectively, both physical property and time-wise. Shortening the time of synthesis, as well as producing more effectively, would highly impact their drug

delivery usage, since it is possible to produce more for less time, and change their physical properties quickly. This kind of method would quickly find its place in the field of pharmacokinetics and drug delivery because it brings new solution that offers easier, shorter, more personalized and effective way to synthesize and produce nanoparticles.

3. MATERIALS AND METHODS

3.1 Designing the microfluidic chip

Microchip represents quite universal unit, but despite its core simplicity, chip design varies a lot for different microfluidic chips with different purpose. Designing the microfluidic chip is very initial and simple step, but also one of the most important steps. All microfluidic chips follow the same concept, with major differences in design correlated with their purpose. Chip is used to mix two or more phases on a microscale, and usually consists two phases, inner and outer phase. Simple concept, which gets more complicated with every single phase or solution introduced into mix. This usually brings new challenges in designing the chip, since more channels have to be introduced, and on a microscale level, this is easier said than done. Every chip is constructed from glass tubes with different diameters (usually represented in millimetres), which represent different channels, inner and outer channel respectively. Each channel will have a certain solution or mixture which will flow through a glass tube and eventually mix with another solution, or create droplets within outer phase.

Design of a microchip starts on a drawing board, and it does include of setting a protocol for chip building, with more abstract and creative approach. Starting point of every chip design is a well established protocol for building the simple microchip with two phases, inner and outer phase. Example of the one of simplest chip designs is shown on picture below.

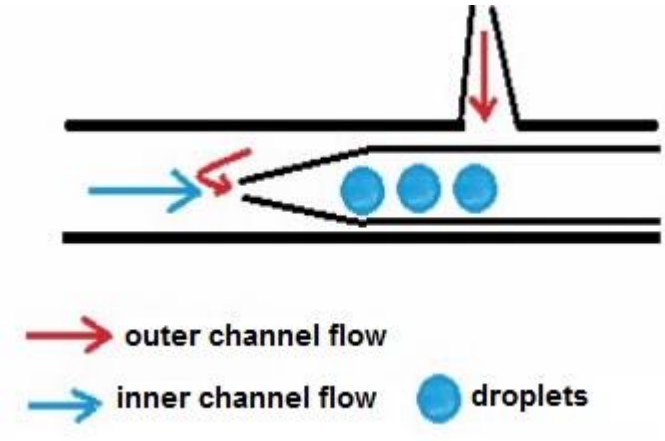


Figure 6. Most simple chip design with its purpose, usually used as a starting point in any more complex chip design

Since glass tubes are the main component of every microfluidic chip, setting their proper placement and position is the main goal of chip design and drawing. Only with this approach it is possible to predict and visualise the behaviour of different solutions that flow through the chip, as well as possible creation of droplets, as well as their size. As aforementioned, Types of microchip may vary depending on its purpose. Some experiments have well established protocols, and chip design is quite straightforward, while other experiments require more innovative design and bring novel and less familiar outcome to the experiment or method itself. Since this particular project required more than two solutions into chip, it was needed to come up with new chip design as well as trying out different chip types.

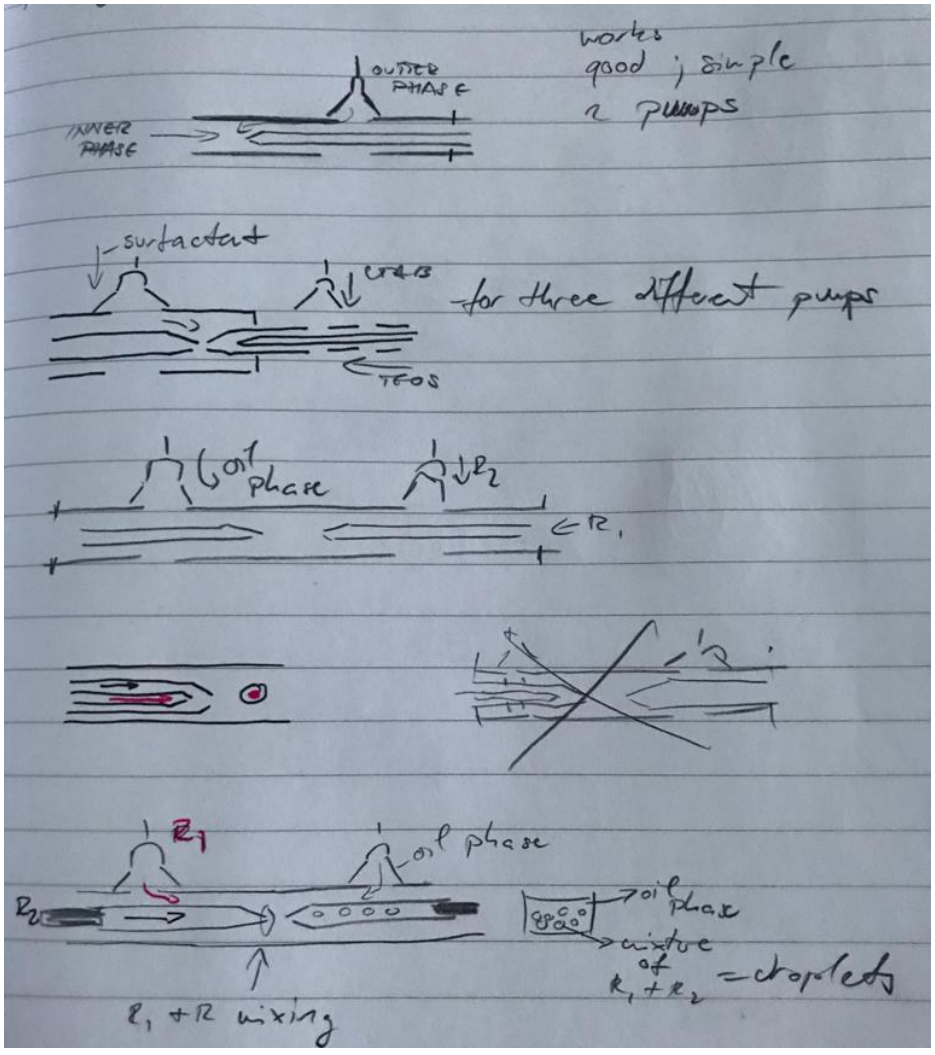


Figure 7. Example of simple drawing board, emphasising the possibilities and ideas introduced into creating every chip, as well as whole process of improving the chip design

As it can be seen from the different chip drawings, there is a lot of trial and error, until a certain chip design is selected to be constructed, and used. After a discussion with co-workers, a small protocol is established for a final design, and chip construction can begin. In picture below, the illustration of the final chip design is shown.

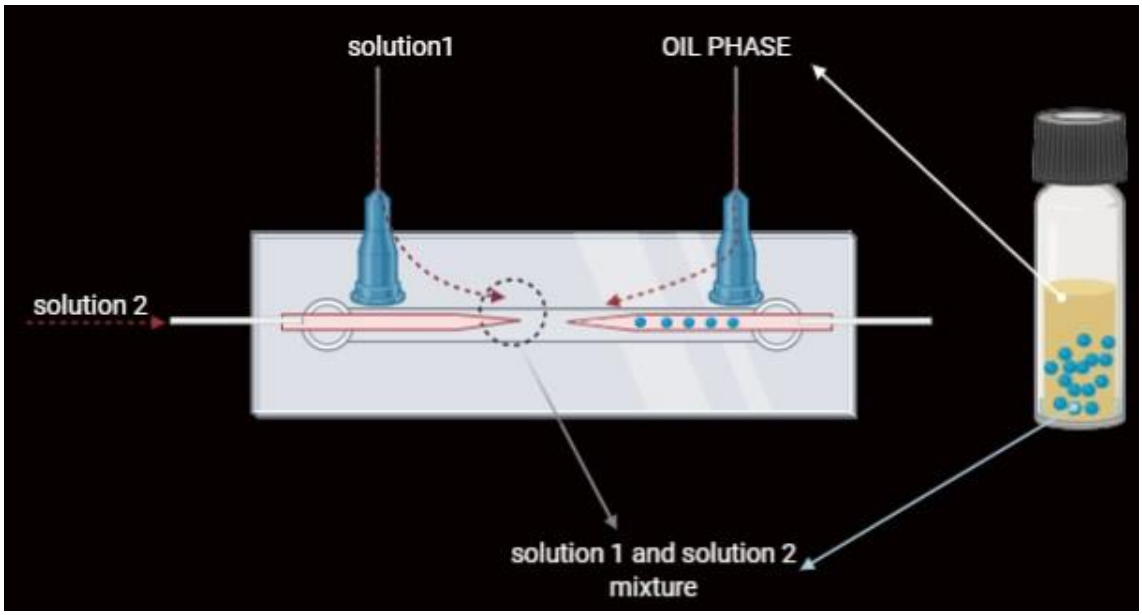


Figure 8. Illustration of final chip design, as well as its purpose and basic idea of its usage

3.2 Construction of microfluidic chip

Contrary to designing part, which is more abstract and theoretical, constructing the microchip is more related to handcrafting. According to blueprint, and set protocol, all the glass tubes need to be cut and put together in order to fit the purpose. First, corresponding borosilicate glass tubes are selected. Inner tube is separated with a PN-30 Magnetic Glass Microelectrode Horizontal Needle Puller by Narishige Japan. This is a device that uses heat source as well as magnetic puller in order to separate borosilicate glass tube in two parts with cone shaped endings, symmetrical for both separated sides. Cone shaped end of each glass tubes require a fine sand paper treatment in order to make a smooth edges and symmetrical opening, where mixing will happen and droplets will form. After this step outer tube is cut with diamond cutter and in places where syringe comes, as drawn in designing process. Inner tube is placed inside outer tube and syringe is put in appropriate place and fixed with mixture of epoxy and hardener. Two metal tubes are placed to fit in the ends of outer tube and fixed with the mixture of epoxy glue and hardener. Chip is left 24h in order for glue to fully dry and thus provide

optimum results when used. It is very important to cut the tubes in right places and clog them with glue, in order to prevent mixing of solutions before predetermined place.

This provides optimum results, and allows easier manipulation of what is happening inside chip. Syringe caps are used to deliver solutions or mixtures to outer channels, and they prove to be very convenient.

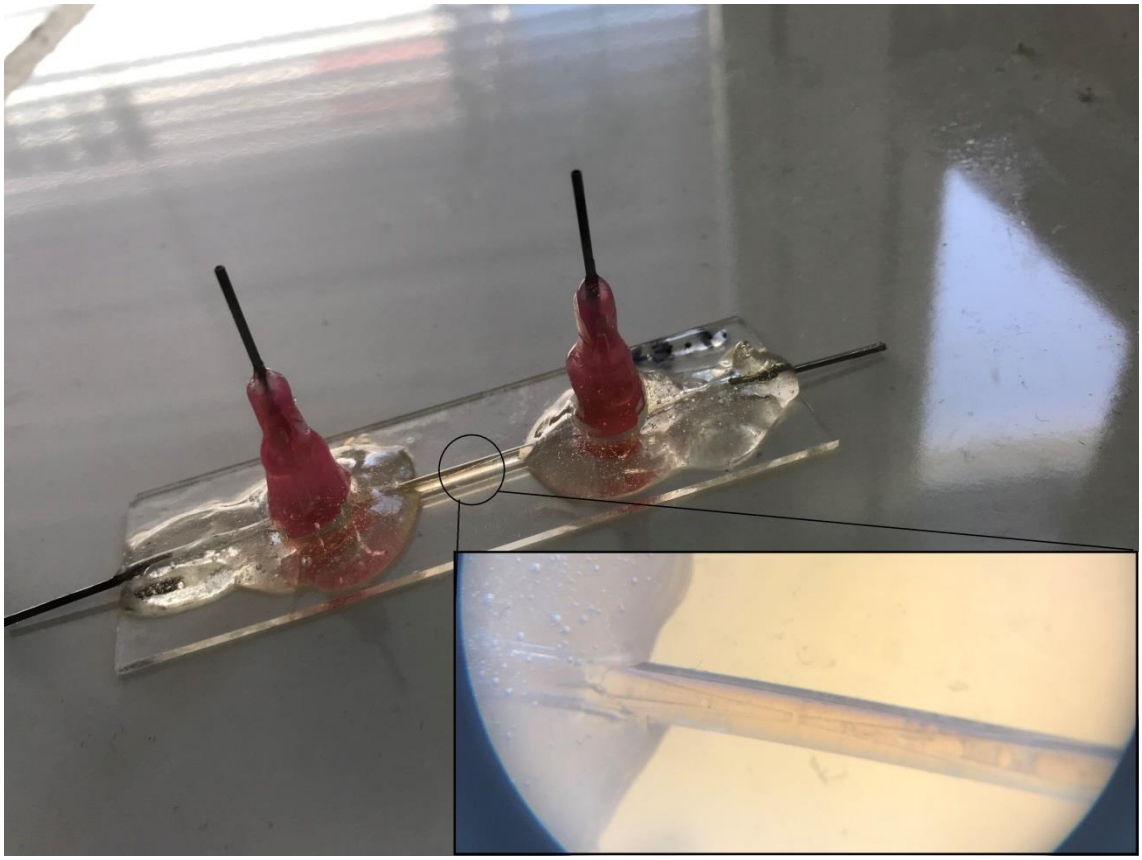


Figure 9. Chip built for this particular experiment with two inner channels, and a closer look to their positioning, shot with light microscope

For this particular chip, borosilacte tubes were used for both inner and outer channels. For inner one, the tube with 1.10mm inner diameter, and for outer one, the tube with 1.56mm inner diameter were used. The difference between these two diameters will provide enough space inside chip for mixture of solutions, as well as for uninterrupted flow of the oil phase. The seemingly small difference between two tubes is vastly important when it comes to controlling the flows inside the chip, thus manipulating the flow speeds, leaving minimum space for possible problems that might occur. The whole chip is constructed in specialised area with PN-30 Magnetic Glass Microelectrode Horizontal Needle Puller, and includes all the tools needed, such as all the glass tubes, diamond cutters, sand paper, epoxy and hardener, syringes, pinchers and forceps. The

chip construction area is placed inside hood, so it prevents dust and external factors to get in.

Whole constructing process is monitored with a light microscope which helps to see the edges of tubes, as well as their placement. A small “chip building lab” is shown in a picture with all the tools mentioned.

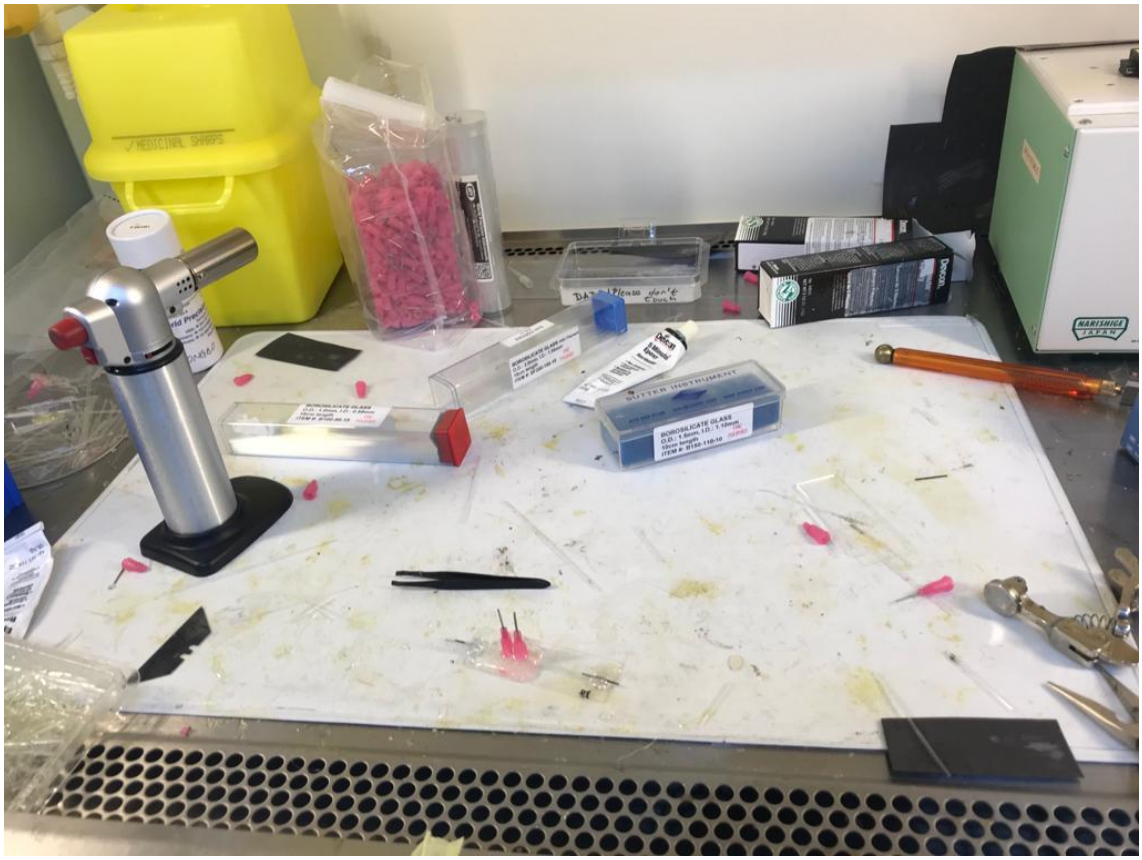


Figure 10. Small workplace under the hood that represents space exclusively intended for chip construction containing all the tools necessary as well as magnetic puller and waste

3.3 Solution preparation for microfluidic chip

This novel method required few different solutions and components used. Its optimisation could be separated in two different parts, with different solutions needed for each optimisation protocol. First one was using a simple two phases, oil and water phases, in order to find optimal settings for droplet synthesis. Idea was to create a most stable water droplets inside the oil phase, and using those settings as a main guideline in creating actual nanoparticles inside a droplet, using more components. Water phase was coloured with a food dye (5ul of food color in a 5ml of miliQ water), while oil phase

represented mixture of span80 surfactant and mineral oil. Oil solution was prepared in three different concentrations: 2% (100mg span80 in 5ml mineral oil), 4%(200mg span80 in 5ml mineral oil) and 6% (300mg span80 in 5ml mineral oil). In order to create stable droplet it was need to make many combinations of different oil phase concentrations as well as fluid flow speeds.

As creating nanoparticles inside the droplet is the main goal of this experiment, it was necessary to use all the right components and solutions that are used in common methods when it comes to nanoparticle synthesis, especially the mesoporous silica nanoparticles. After using the simple oil and water phase, and setting up a protocol for droplet synthesis, it was time to introduce all the components used in MSN synthesis, and establish their concentrations in order to create stable droplets, and create a new protocol. Principle is similar to first optimisation step, but more complicated, since it uses more solutions and mixtures, thus giving more options to be adjusted, such as concentration, and of course, fluid flow speeds. This part uses also two different phases, water and oil phase, where water phase is a mixture of few different solutions, and its mixing is happening inside a chip. For oil phase, Span65 was used, but since it had very low to none solubility in mineral oil, oleic acid was used. Concentrations used for Span65 phase, were same as for Span80 in first optimisation, meaning oil phase was prepared in three different concentrations: 2% (100mg span65 in 5ml oleic acid), 4%(200mg span65 in 5ml oleic acid) and 6% (300mg span65 in 5ml oleic acid).

Water phase required two channels inside the microchip, where two solutions would mix inside a chip, as shown in chip design illustration, and create a water phase which would, finally produce droplets inside the oil phase. There are two main components in water phase, TEOS (Tetraethilsilicate) and CTAB (Cetyl Trimethyl Ammonium Bromide). All of these components are commonly used in bulk methods of MSN synthesis. The basic idea behind using these two components as main ones is that inside a chip, CTAB creates a template in water. This template occurs under alkaline pH and it is in form of micelles. This solution would further mix with TEOS solution inside a chip. TEOS is very reactive to water, and it precipitates upon encountering water phase. Within the mixture, precipitated TEOS should deposit over the CTAB micelles. All of this should happen within water phase, which creates droplets inside a span65 phase, thus having many templates with TEOS precipitate around them, inside each droplet, where droplet has a role as a microreactor. In order to prove this theory in practice, it

was necessary to establish concentrations of both CTAB and TEOS, and other components that would make solutions of the main two components for the water phase. After many calculations, it has been decided to mix TEOS with ethylene glycol, as well as trying to use it just as a TEOS, as it is highly reactive with water, and to prevent precipitation from happening before it mixes with water phase. Furthermore, solutions prepared for optimisation were stock TEOS solution, and solution of 45ul of TEOS inside 833.33ul ethylene glycol. For a water phase, there were several options. There were two different concentrations of CTAB in a MiliQ water, including 15mg of CTAB in 5ml MiliQ, and 32,5mg of CTAB in 5ml MiiQ. One more solution was added to the mix, as a third option, and that was 32,5mg CTAB with 151,389ul of Ammonium hydroxide in 5ml MiliQ.

It was necessary to set up different solutions and their concentrations, in order to get the optimal results during optimisation, and predict the mixing behaviour and droplet formation inside a chip. A variety in concentrations of each component introduces more options in the optimisation step, as well as more solutions in creating a final product.

Core chemicals used: CTAB as Hexadecytrilmethylammonium Bromide by Sigma Aldrich, Germany; Span65 for Synthesis by Sigma Aldrich, Germany; Tetramethyl ortosilicate by Sigma Aldrich, Germany, amongst which are other chemicals used to dilute the core components or bind with them, such as Oleic acid, Ammonium hydroxide, 99% ethanol, miliQ water with pH of 6.9 and Ethylene glycol.

Figure 11. Main components used in optimisation protocol, with a main goal of mesporous silica nanoparticles synthesis

3.4 Optimization of microfluidic protocol

Optimization is a most complex step, and most time consuming. It is a point where all of previous steps come together. Since it is a novel method, optimization and protocol writing requires most time, and it is based on trial and error methods, because it needs to be done from the grassroots. The reason behind optimisation complexity is a big number of variables and combination included. In this step, all of the prepared solutions and concentrations are put into syringes attached to the syringe pump system that will inject solutions into the microchip. Injecting the solutions is done with PHD Ultra Advanced Programmable Syringe Pump by Harvard Apparatus. These fluid flow pumps offer very accurate manipulation of fluid flows within each channel inside microchip. All phases were stored inside BD Plastic Pak 5ml Syringes during this process.

Figure 12. Harvard Apparatus PHD Ultra Syringe Pump

These pump offer easy real time adjustment of each syringe fluid flows. This feature is crucial because it allows changing of the settings on the go, as well as pausing the process and restarting it if somethings goes wrong. Harvard Apparatus PHD Ultra Syringe Pumps have been proven as a good choice, because they introduce almost infinite number of variables, allowing to change fluid flow speeds for each pump, as

well as being compatible with different capacity syringes, such as Becton Dickinson Plastipak syringes ranging from 1ml to 20ml volume capacity. Pumps also allow different settings in a volume per time scale, which mean it is possible to set up a fluid flow speed for one syringe in microliters/hour and other in millilitres/minute, according to targeted purpose. This future is crucial in setting an optimisation protocol, and brings many different solutions and opportunities, as well as affecting the protocol in real time, and changing it on the go.

The optimization process was separated into two major phases. First one was writing optimization protocol for creating stable droplets, and it included simple two channel chip, as well as water phase with dye as a constant, and three different concentrations of oil phase. The goal of this first, less time consuming optimisation, was to get basic idea of which fluid flow speeds to use in order to create stable water phase droplets inside an oil phase. This was necessary in order to get basic idea on what fluid flow speeds to use in major goal of this experiment, which is creating nanoparticles inside these droplets. First optimisation was expected to bring major guidelines on how to set up whole system for three solutions, and within more complex microchip. The idea behind second optimisation step, one that took major time of whole experiment, was using knowledge from the water/oil phase optimisation and applying already familiar settings in order to mix TEOS and CTAB and creating those droplets inside span65 and oleic acid solution. All of the optimisation steps were monitored by high speed camera microscope at all times. This allowed monitoring of mixing, and droplet creation, in real time and all the time. Also, this microscope was useful in taking the images of droplets within the sample, which were used in the image analysis of droplets, in order to determine their physical properties. Microscope camera used in this experiment was High Speed Digital Microscope camera by Meros, Dolomite Microfluidics, United Kingdom. This camera is designed to suit the purpose of monitoring microfluidics and recording droplet production in microscale at high speeds, which makes it ideal for this type of experiment.

3.4.1 Optimisation protocol for creating stable droplets

For this step, the simple chip was used, same one as illustrated in the chip design section. Since this step included only two phases, water and oil phase, simple chip was suitable to use in this optimisation step.

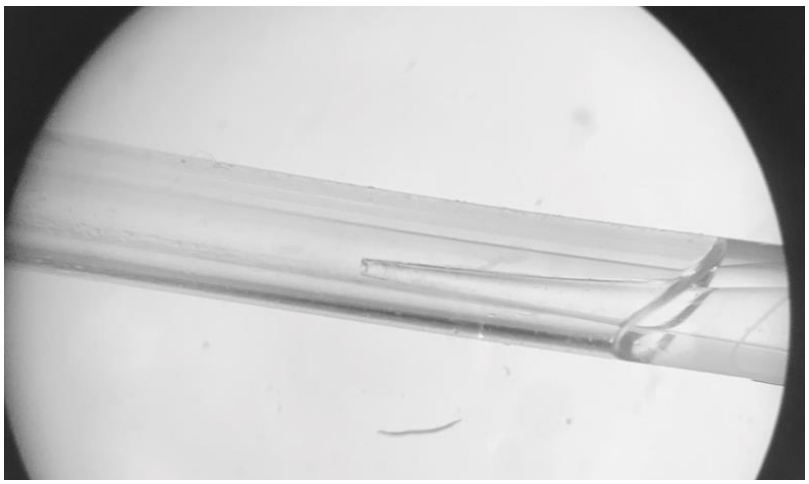


Figure 13. Simple chip with inner and outer channel used in first optimisation step, imaged under light microscope

Three constant fluid flows for each were determined. For outer phase, 1ml/hr; 2ml/hr and 5ml/hr are used for every solution, 2%, 4% and 6% respectively, while for inner phase fluid flow speeds of 100ul/hr;200ul/hr and 500ul were used. All of the inner channel speeds were combined for each of three of outer channel speed, as well as every single oil combination. This resulted in a collection of 25 samples in total, and after imaging those samples it was possible to see which fluid flow settings do have optimum results for creating stable droplets. Although this was just an introduction for the next optimisation step, it is very important because it determines the starting point for the core optimisation of the whole experiment, which in a novel method, such as this one is vastly important. By completing this step, it was easier to predict and determine the most optimal settings for synthesising MSNs inside a droplet.

3.4.2 Optimisation protocol for creating nanoparticles inside droplet

Setting up second optimisation protocol, in order to create a stable droplets as in first part, but with numerous nanoparticle templates and precipitate around it, and all inside a single droplet, was the most important step of the whole experiment. Since it is a novel method, there were no previous guidelines on how to start optimisation, except the observed results from previous optimisation. The results from protocol on creating stable droplets introduced a helpful outline on how and where to start this optimisation protocol. In theory, it was quite simple to predict the mixing of solutions inside a chip, and behaviour of the created droplets, but in practice, it had proven to be whole different story. Due to many possible combinations, and possibility to adjust all the

settings, this step was proven to be most time consuming and most trial and error consisted step.

Since this chip required three syringes with three different solutions, the whole setup required to be upgraded compared to the first optimisation. As far as technicality goes, three Harvard Apparatus PHD Ultra Syringe Pumps were needed to operate at the same time, each one carrying different syringe. All three pumps were connected to the chip, while whole process was monitored by high speed camera microscope. This microscope camera records the process in microscale in real time, and offers recording and image taking without interrupting the process. This feature was of vital importance when it comes to adjusting the protocol, because it allowed adjusting to be fluent, and didn't require stopping or pausing the process.

Figure 14. Setup used for optimisation and synthesis, consisted of different syringe pumps and high speed camera microscope

Hardware settings for this protocol are clearly determined, and they include three syringe pumps: one with TEOS mixture, one with CTAB mixture, and one with span65 oil phase. All three are syringes are connected to corresponding channel on the chip. Span65 is exclusively connected to the outer channel, as it represents the outer/oil phase. Initially TEOS was connected to the outer channel as well, while the CTAB was connected to the inner channel. This way CTAB and TEOS would ideally mix and then create a water phase which would form droplets by being pushed into second inner

channel by oil phase. During the optimisation, CTAB and TEOS were switched, meaning syringe containing TEOS was moved to the inner channel in order to minimise TEOS contact with any other compound before mixing with CTAB, due to high reactivity of TEOS. Basic premise of this experiment was to mix CTAB and TEOS solutions on the microscale, inside the chip, where inside this mixture CTAB creates micelles in water, and TEOS precipitates when in contact with water. Precipitate would stick around the CTAB template, creating desired shape from silicate, thus forming nanoparticles around the template. All of this would happen within the each droplet that is formed inside the chip by being pushed through inner channel by oil phase, and creation of droplet happens due to different HLB factor and viscosity.

In order to accomplish this, it was necessary to determine proper fluid flow speed for each solution respectively, thus creating a clear protocol containing most optimal settings in order to create stable droplets. This is done by combination of different solution concentrations and different flow speeds for each solution, which creates an excessive number of possible combinations. The process of optimising this step is explained further in text.

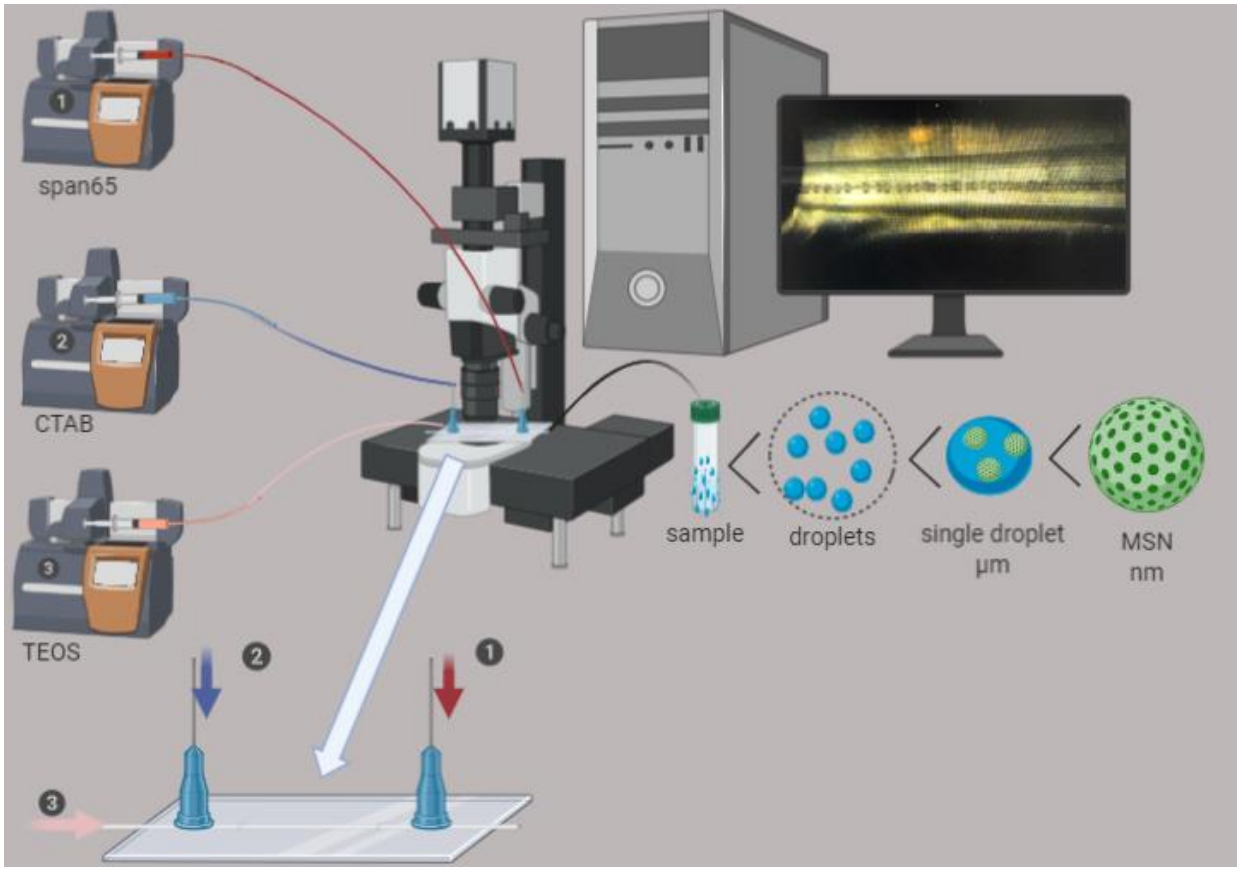


Figure 15. Illustration of the whole optimisation step, as well as the picture of forming the stable droplets caught in real time with high speed camera microscope

As fore- mentioned, optimisation was consisted over trial and error methods, as well as non-stop monitoring of the process. Starting step was using fluid flow speeds acquired from first optimisation step, as a guideline, and correcting these settings on the go in order to create a stable droplets. Fluid flow speeds for CTAB and TEOS were in a ul/hr, while speeds used for an oil phase, thus Span65 were used in a ml/hr. Significant increase in a speed between two phases was in order to assure that oil phase will push water phase mixture through inner channel and create a sustainable droplets. Speed of each solution affects behaviour of fluids inside a chip, and can be adjusted individually for each solution. All of the parameters are adjusted one at the time, and formation of droplets is observed. As soon as droplet formation becomes constant over some time period, usually more than 10mins, sample is collected and will be prone to further analysis. By this method, numerous samples were collected, and all of settings used for each samples are stored in database. Each sample goes through certain time points, in which is observed if droplets merge or not, thus being able to see if droplets are stable or not. If they are preserved more than two hours, sample is taken for imaging and further analysis, because each droplet should contain thousands of MSNs, depending on a droplet size.

3.5 Sample collection

As for the first part of optimisation, after all of the combinations of fluid flow speeds and concentrations, 25 sample vials were collected. Each one of them was imaged with a microscope camera and droplets were imaged in order to see their physical properties. Every single sample was stored for a certain time period, and its droplet formation and stability was checked. All of the samples were checked in a two hour, four hour and twenty-four hour time scales. It was crucial to determine if droplets remained stable and maintained the initial structure

For the second part, similar principle was followed. Since these samples contained more solutions and chemicals in the water phase, further work was required during the sample collection. Upon droplets being collected inside a 5ml glass vial, there was a lot of precipitate in each sample as well. Because of this, immediately upon collection, a small amount of sample was collected with pipette, and imaged with microscope camera, in order to check if there is only precipitate or droplets as well.

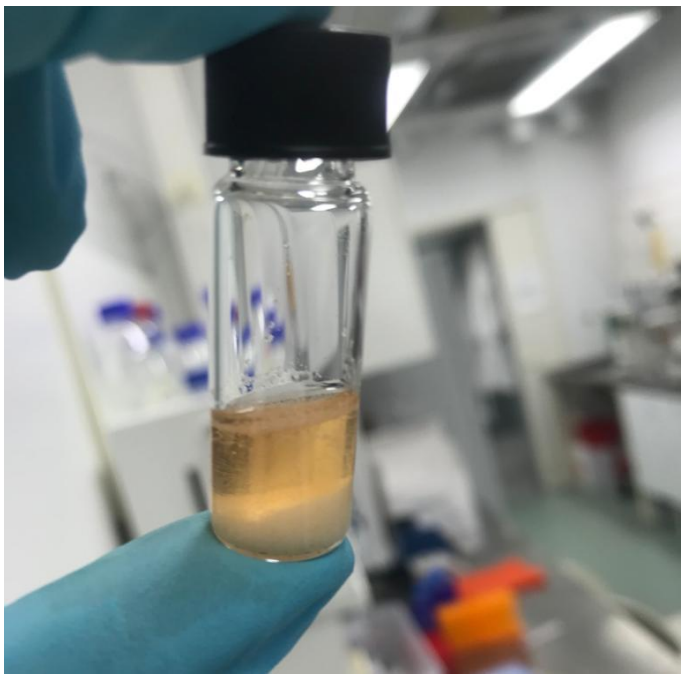


Figure 16. Sample of droplets containing CTAB/TEOS mixture inside a span65/oleic acid phase

By using this practice it was possible to determine which samples are kept and observed through time points, and the ones containing only precipitate were discarded, as well as their settings from optimisation were written down as not suitable for droplet creation. All the samples will be imaged and analysed, and the ones containing stable droplets are prone to further imaging techniques and analyses.

Sample collection is established protocol: collection of sample in 5ml glass vial for given settings, pipetting few microliters of sample and observing it under high speed camera microscope, repeating droplet formation monitoring through different time points, if droplets remain stable, samples go to further analysis for which there is another established protocol.

3.6 Sample washing and preparing MSNs for imaging

This step is exclusively reserved for the second part of optimisation and samples obtained during this process. Only samples that maintained droplet formation for more than two hours are prone to this protocol. Before the protocol, samples usually sit for four hours, this is estimation of time needed for TEOS to create a silicate shape around CTAB micelle templates. Ideally in each droplet by this time there should be formed thousands of CTAB micelles under the alkaline pH of miliQ water, and TEOS, also by encountering miliQ water, precipitates in silica form around the templates. This would

mean that every single droplet has a role of microreactor, in which there are numerous MSNs created, but still holding a CTAB template. Knowing this, it is clear that in order to get the nanoparticles, it is necessary to get rid of the surfactant template. Ironically, in order for washing to be done, droplets created need to be broken apart. It was crucial to have stable droplets for few hours in order to give enough time for precipitate to form, as well as to create a shape around the surfactant template. Main goal of this protocol is to break droplets to acquire the product stored within, as well as washing the product from all of the solutions and chemicals that occurred during the synthesis. By doing this, product is left pure and ready to be analysed and imaged, in order to check the success of the all previous steps. This protocol is established and follows up through two phases, preparing the sample and analysing the sample. For sample preparation, it is necessary to remove oil phase with Single Channel Manual Pipette by Rainin, US. Pipette models used are Pipet-Lite XLS Pipettes with different volume range, depending on amount of oil phase that needs to be extracted. For removing oil phase were 10-100ul and 1-10ml volume Pipet-Lite XLS pipettes were used. After the maximum possible amount of oil is extracted from the vial, the droplets and precipitate are left in the sample, and it needs to be washed. Washing is done with the mixture of 20% ammonium hydroxide solution in ethanol. This solution is prepared by taking 5,7ml of stock ammonium hydroxide solution and mixing it with 4,3ml of 99,5% ethanol in order to get 10ml of 20% ammonium hydroxide solution. In order to wash the sample, 1ml of this solution is needed before vortexing and centrifuging the sample. High concentration of ammonia will react with surfactant template, CTAB in this case, and help wash it out so only silica particles are left. Washing is consisted of adding 1ml of ammonia solution to the sample, which is vortexed, and then put in centrifuge for seven minutes. This step n repeated three times, where after each centrifuge cycle, supernatant is removed, and new 1ml of ammonia solution is added. After this, sample will be taken for DLS analysis.

Figure 17. On the left picture there is DLS reader and vortexer and centrifuge on the right

DLS stands for dynamic light scattering, and represents technique used to measure size of particles, as well as their potential, all by using properties of light scattering. DLS analyser in this experiment was Zetasizer by Malvern Panalytical, United Kingdom.

Taking the certain amount from the sample in a vial and adding miliQ up to 1ml and analysing it with Zetasizer will provide necessary information on whether there are nanoparticles formed in the sample as expected, as well as their size and range. If the analysis shows there are nanoparticles synthesized, and they range between certain nanometer range, sample goes for further analysis, where actual MSNs synthesized in process will be imaged using the TEM (Transmission Electron Microscopy).

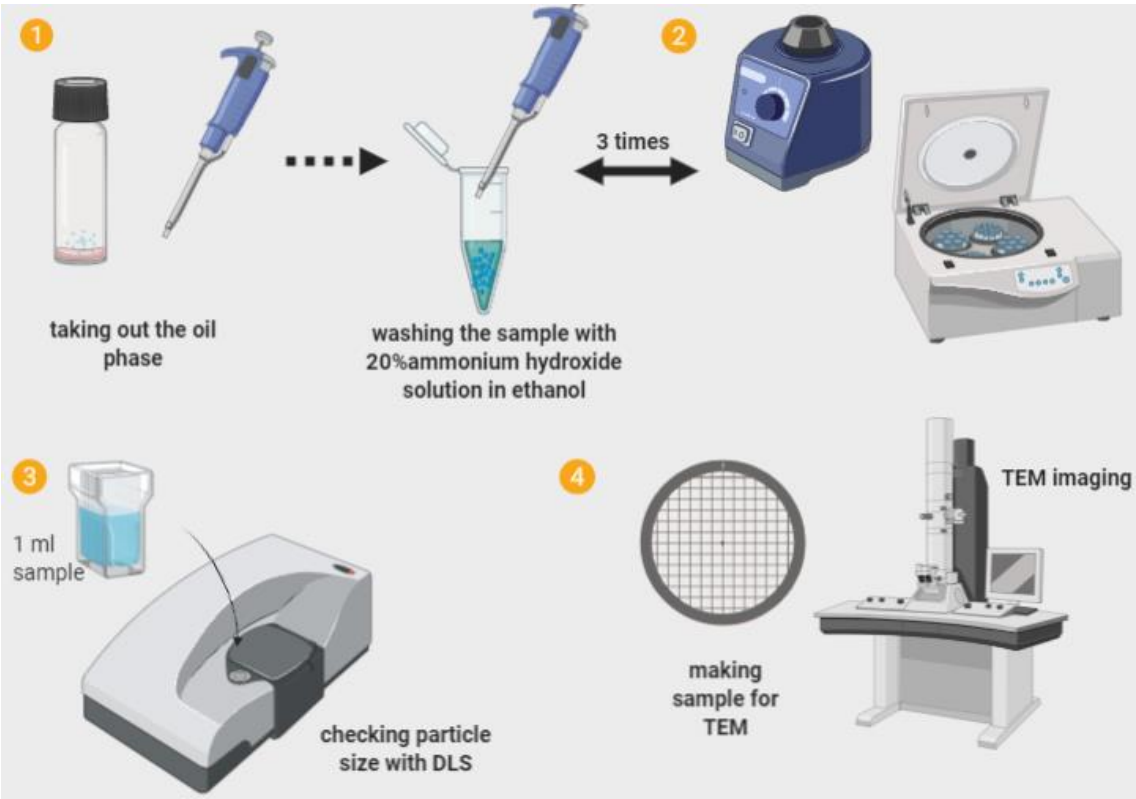


Figure 18. Detailed llustration of sample washing protocol as well as further analyses that sample is going through

3.7 Transmission Electron Microscopy

As this is the novel method, it requires constant monitoring and imaging. As fore-mentioned, a droplet creation and produecd droplets are monitored and imaged at all time by digital microscope, and light microscope is used for chip construction. as the main goal of the experiment is to create a nanoparticles, Mesoporous Silica Nanoparticles to be exact, a special imaging device will be necessary to check and image the sample since it is in a nanoscale. In order to do this, and obtain proper image that are able to show the nanoparticles, and their physical properties, it is necessary to use a Transmission Electron Microscope (TEM). TEM possesses bigger resolution than other microscopes used because it uses electron beam which penetrates through specimen in order to form an image. In this way, it is possible to image nanoparticles and more important, their detailed topography and properties, which is of major importance, since the physical properties of MSNs are crucial for its use.

As stated in a previous protocol, sample that holds synthesised MSNs is prone to go through washing and DLS analysis steps before it is taken for TEM imaging. This will help to know if there is possibility of nanoparticles inside a sample, and is there a purpose for Transmission Electron Microscopy. After all the steps from sample washing

protocol, sample needs to be put on a metal grid. Possible nanoparticles are still in a suspension, which is put on a grid and dried, after which it is taken to imaging facility and will be imaged with TEM, and if images prove there are MSNs, images will be analysed in order to check the products physical properties.

3.8 Image Analysis protocol

As mentioned before imaging of the droplets will be done with high speed microscope camera with magnification 1. For image analysis, software called ImageJ by Fiji will be used, as well as certain steps of image analysis within this software in order to get relevant and constant data for each sample. After trial and error methods, the image analysis protocol has been established and it was consisted of few steps in order to compensate for noise factors such as not even pixel values of background due to different lightning within images. The protocol was consisted of these steps, in exact order: Opening the image>convert image to 8-bit> duplicating image> Gaussian blur filter (radius:100) to duplicated image> image calculator: subtract original image from duplicated one> manual threshold> analyse particles (area size 300-Infinity(px); roundness:1)> show outlines.

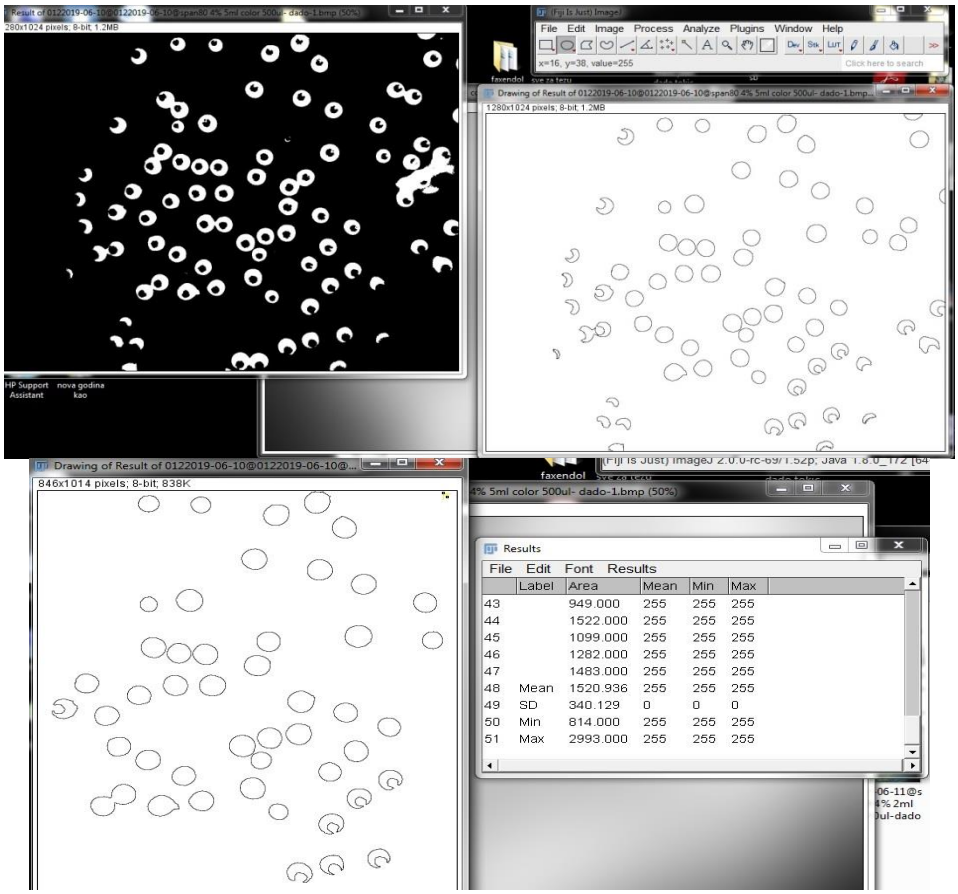


Figure 19. Screenshots from one of the analyses, with fore mentioned protocol steps, as an example

Particle size is measured as area in pixels. It is possible to have a scale in micrometres for example, and measure the scale in pixels, so that one unit of micrometre corresponds to few units of pixels, in order to interpret results easier and more familiar unit.

When it comes to the imaging and image analysis of the second optimisation protocol, same imaging analysis protocol can be used, with some minor exceptions. Reason behind this lies in a greater amount of precipitate in each image of droplets taken during the second part of optimisation. This phenomenon vastly affects imaging and image analysis because it creates a lot of noise and artefacts that can affect the data that will be analysed. In order to cope with this problem, it was necessary to add minor tweaks to the aforementioned image analysis protocol, and this would be applied only for images where there is significant amount of precipitate that will affect image analysis. One method proved to be useful when it comes to coping with excessive amount of precipitate and that is applying the Laplacian filter during image analysis. Laplacian filter represents the 2 D measure or kernel, often applied to Gaussian filter, and it is used in order to improve edge detection. It is used with the Gaussian filter, where its use lies in the reducing sensitivity of Gaussian filter to noise. This proves as extremely useful method, because precipitate can be treated as noise, and as it mostly occurs very close to particles, it does affect the imaging and image analysis, in a way it may change a shape of particle during analysis, thus bring false results when it comes to extracting data. A seemingly small step, but of major significance when it comes to the image analysis of samples taken during the second part of optimisation.

4 RESULTS

3.2 Creating stable water phase droplets inside an oil phase

In this section images will be shown, and from different phases. Images from sample collection, images of droplets, as well as image analysis and table with average size of droplets, depending on production parameters. These table will represent quantitative results.

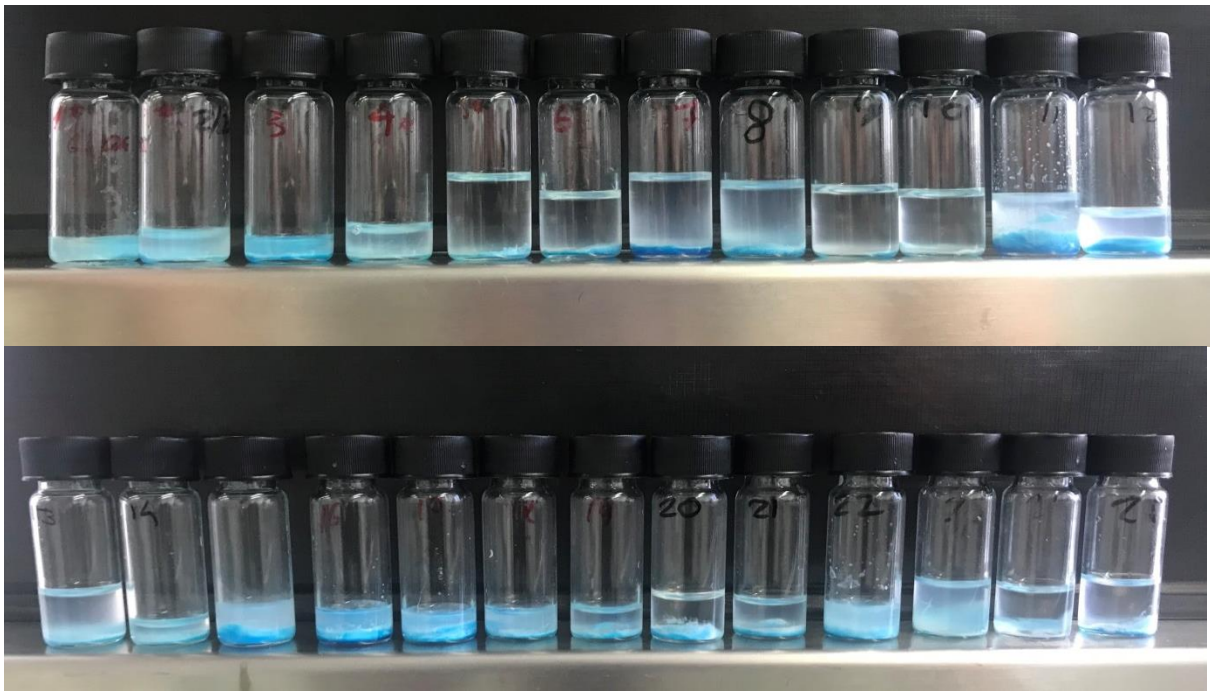


Figure 20. 25 different samples collected, each containing droplet produced with different settings

	span 80 2%	1ml/hr	2ml/hr	5ml/hr
DYE	100ul/hr	sample 1	sample 4	sample 5
	200ul/hr	/	sample 2	sample 6
	500ul/hr	/	sample 3	sample 7
	span 80 4%	1ml/hr	2ml/hr	5ml/hr
DYE	100ul/hr	sample 10	sample 13	sample 14
	200ul/hr	sample 9	sample 12	sample 15
	500ul/hr	sample 8	sample 11	sample 16
	span 80 4%	1ml/hr	2ml/hr	5ml/hr
DYE	100ul/hr	sample 19	sample 20	sample 24
	200ul/hr	sample 18	sample 21	sample 23
	500ul/hr	sample 17	sample 22	sample 25

Table 1. Table that shows fluid flow speed settings used for every sample for both oil, and water phase

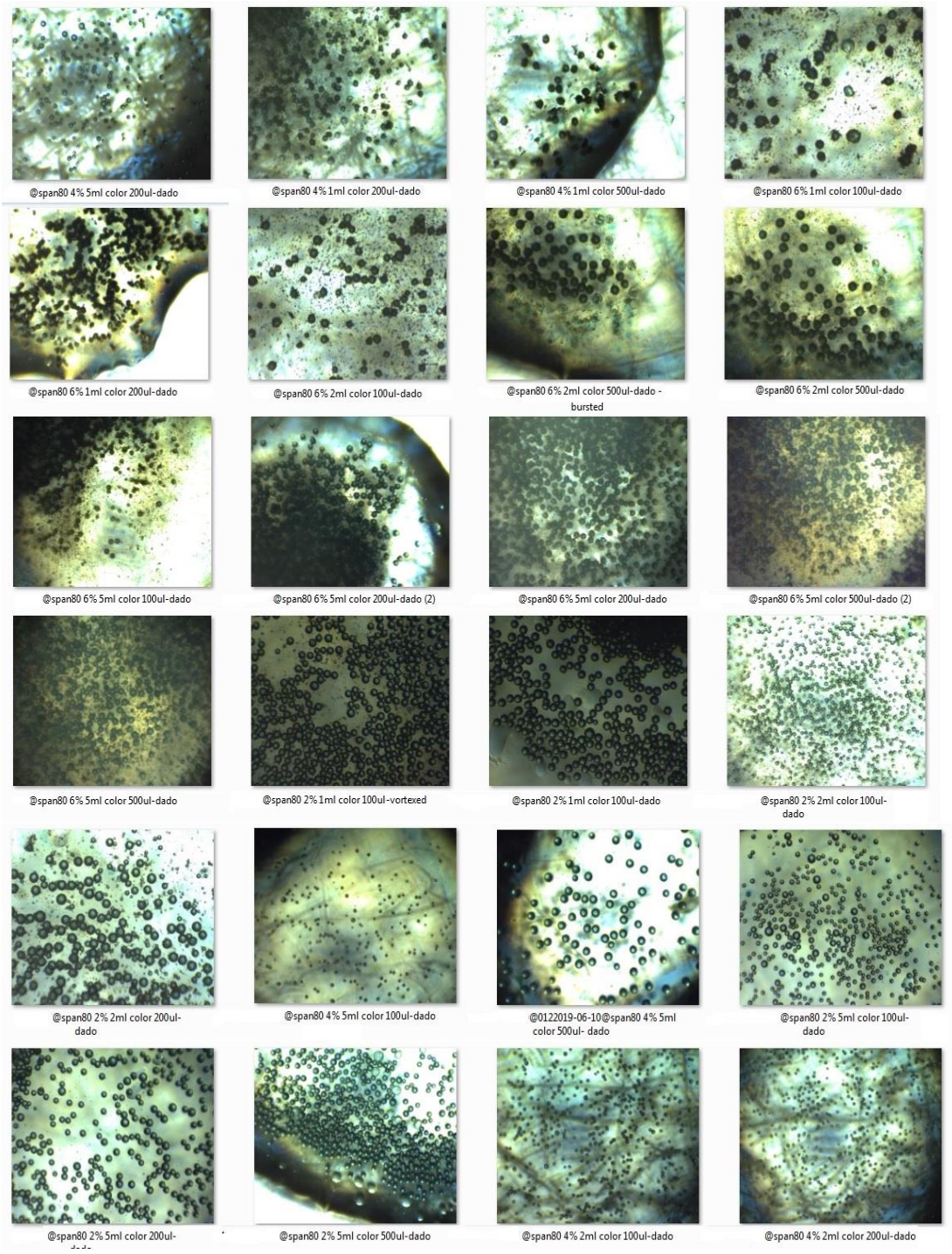
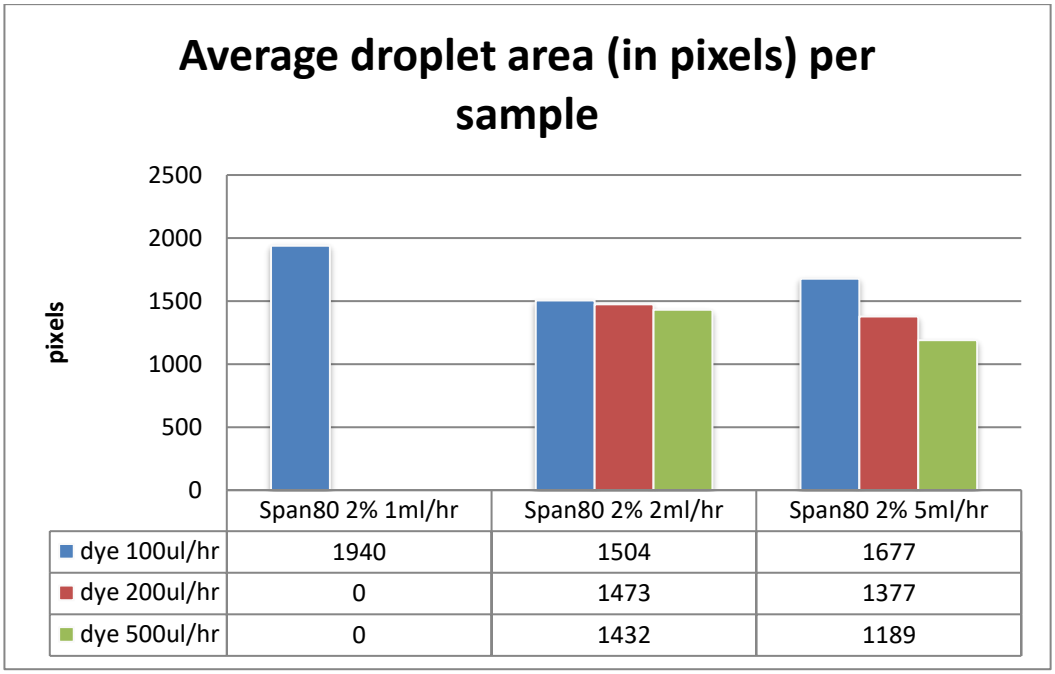
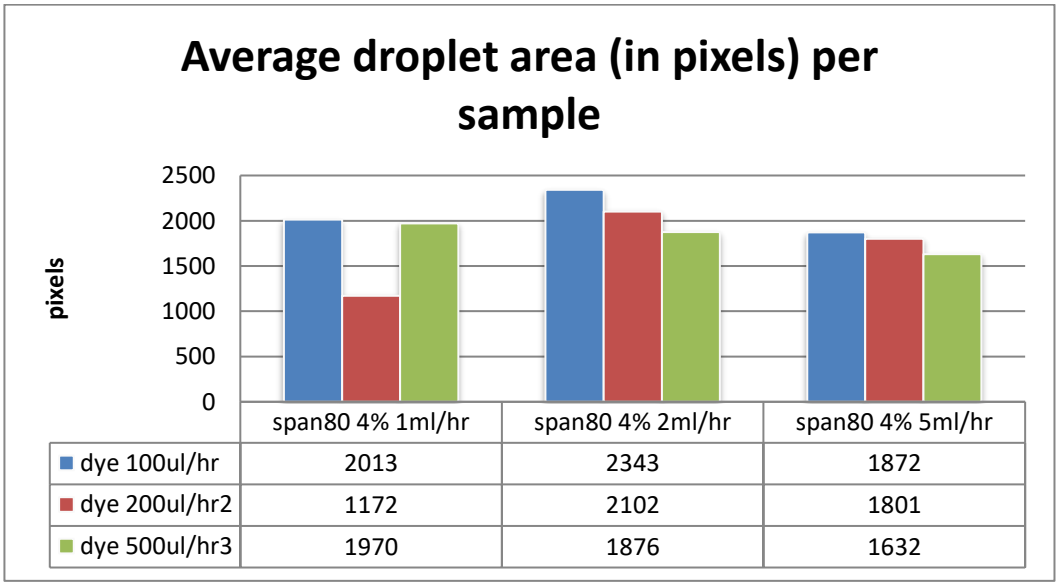


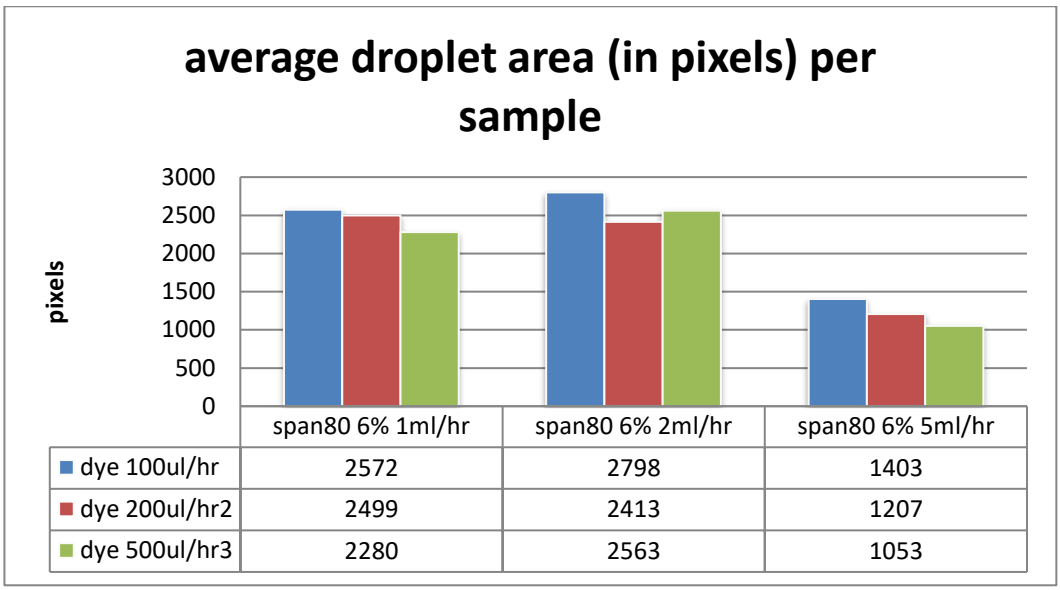
Figure 20. Images of the all the samples taken from creating stable droplets protocol, taken with High speed Digital Microscope. Each image contains concentration and speed of two phases used to create shown droplets. Some samples were vortexed before imaging due to high concentration of droplets in certain samples. From images it is possible to see stable water phase droplets, their difference in size and properties correlated to different optimisation settings. After image analysis, it will be possible to determine the size of droplets for each sample, which can be used as guideline for further research and experiments, such as synthesizing nanoparticles inside a droplet.



	Span80 2% 1ml/hr	Span80 2% 2ml/hr	Span80 2% 5ml/hr
dye 100ul/hr	1940	1504	1677
dye 200ul/hr	0	1473	1377
dye 500ul/hr	0	1432	1189



	span80 4% 1ml/hr	span80 4% 2ml/hr	span80 4% 5ml/hr
dye 100ul/hr	2013	2343	1872
dye 200ul/hr2	1172	2102	1801
dye 500ul/hr3	1970	1876	1632



	span80 6% 1ml/hr	span80 6% 2ml/hr	span80 6% 5ml/hr
dye 100ul/hr	2572	2798	1403
dye 200ul/hr2	2499	2413	1207
dye 500ul/hr3	2280	2563	1053

Table 2. Each table above show the size of particles for each sample taken, presented in pixel areas. This is easy to convert to metric unit by assigning the measure line of 1micrometer that corresponds to certain number of pixels. It is shown how the fluid flow speed affects the droplet size.

As the tables and images above show, it was possible to create droplets using the optimisation protocol. After using different settings, droplets with different properties were created, and imaged with same technique. Image analysis allowed us to interpret these properties and their differences with numbers, and compare them. It is easy to observe how different settings affect size of the droplet. Trend of results showing droplet area in pixels can be observed through two different parameters, concentration of the oil phase, and fluid flow speed. From images it is clearly visible, that lower concentrations of the oil phase, 2% and 4% span80 in this case, are more suitable for droplet production than 6%. This means that 6% span80 oil phase is too thick and too viscous for water droplets, and makes imaging harder as well, because oil phase viscosity directly affects the brightness of the taken images. Regarding the fluid flow speeds and their variations, droplet size follows certain trend with different combinations of fluid flow speeds, with minor deviation in the results. These deviations are expressed in a way where particle is bigger or smaller than expected, which may be the consequence of imaging or image analysis as well. What can be concluded from analysed size of the droplets, it is clear that fluid flow speed and droplet size are inversely proportional, meaning the higher the fluid flow speed is, the lower droplet area is. This can be explained that higher speed creates higher force of the water phase

exiting the capillary, thus creating more droplets at the time, meaning their area is smaller due to high speed production.

4.2 MSN synthesis using the microfluidic chip

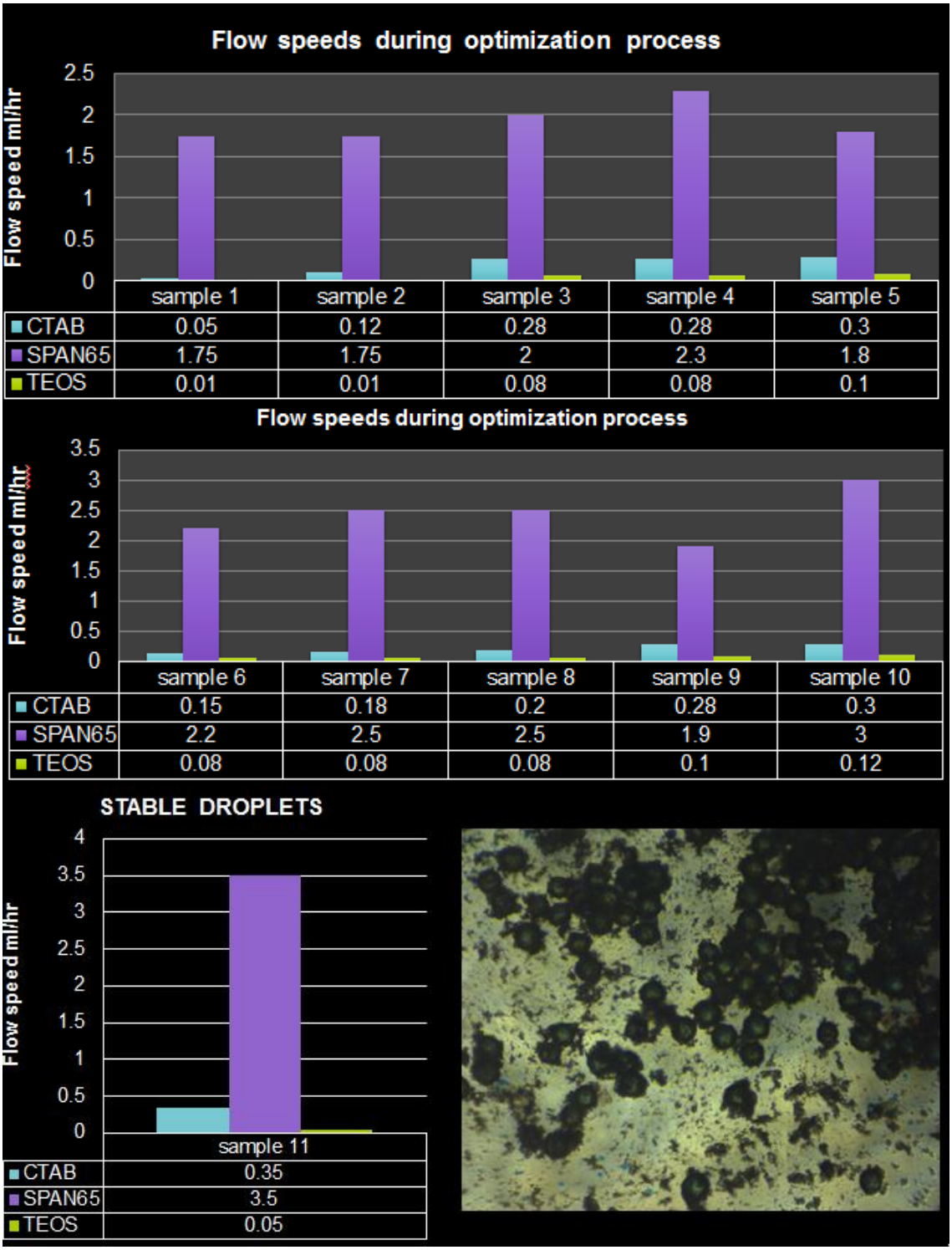


Table 3. Tables that show ratio of fluid speeds used in order to produce droplets with nanoparticles inside, as well as example of setting used in creating stable droplets

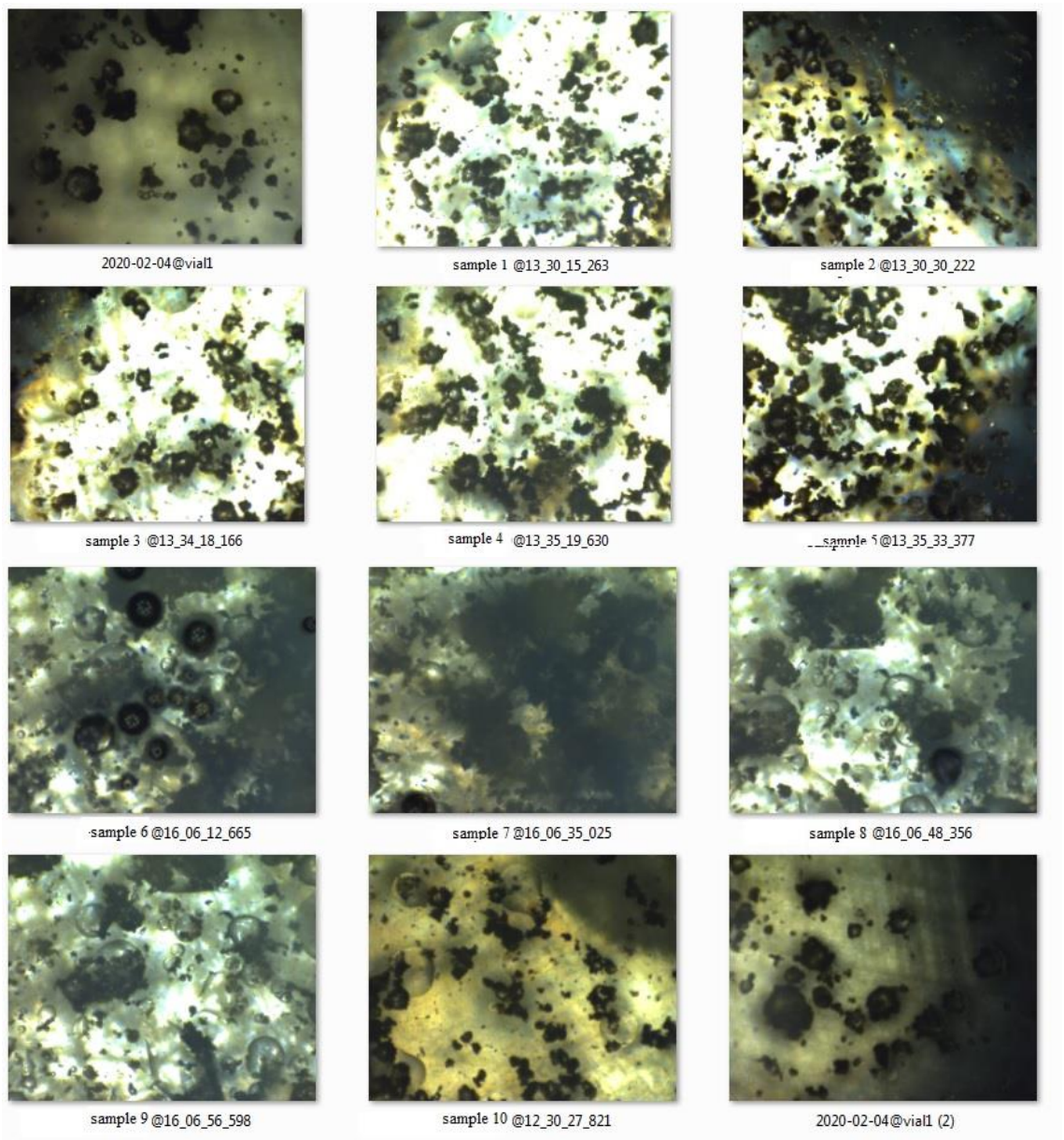


Figure 22. Images of ten samples collected using fluid flow speeds as stated in tables, shot by high speed digital microscope camera

As it can be seen from comparison of images between two optimisations, it was quite challenging to produce stable droplets in a first place during the second part. Ten samples were collected, each one of them containing droplets and a lot of precipitate. Main problem was that droplets were not stable and would burst or merge within one of the time points. Only one sample had fully stable droplets, as well as not excessive amount of precipitate, and it was sample 11, as stated in a table or pictures labelled vial1. This sample was imaged within certain time points, and shown to have stable droplets, which means it was prone to the further analysis including dynamic light scattering and imaging.

4.3 Dynamic light scattering analysis

Figure 23. In the tables above the result from DLS are shown, acquired by Zetasizer. In the first table it is visible that sample was measured three times, and the main parameters measured were number of particles, volume and intensity, all presented in diameter within a nanoscale. On bottom image, all of the setting used for dynamic light scattering are shown, such as dispersant, temperature, count rate, duration, position of measuring, as well as what cuvette u volume used. The graph shows size distribution by volume, where it is clearly visible that there were particles inside a sample, and their size ranged around 100nm. Zetasizer has calculated an average diameter in nanometres, which was 166,4nm for this sample.

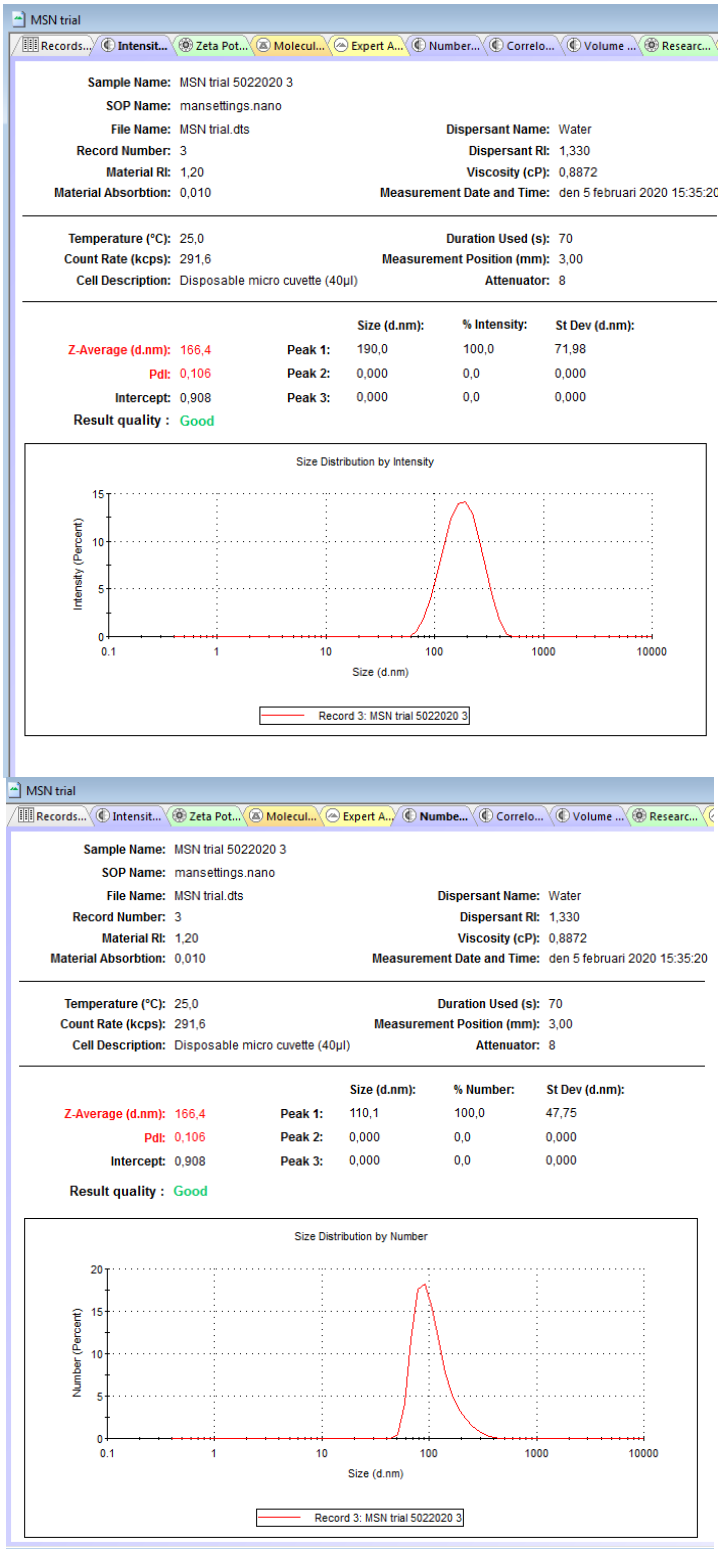
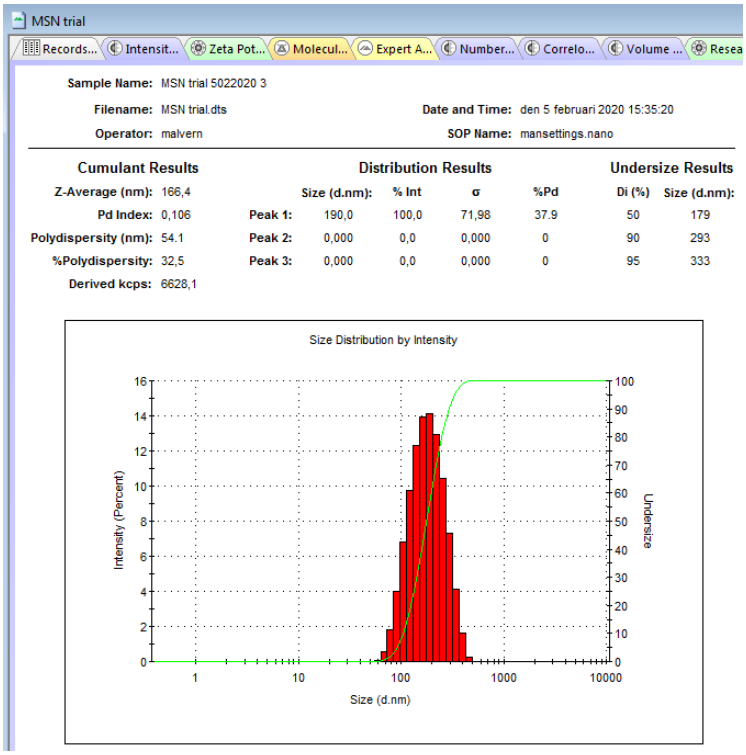


Figure 24. Two graphs above show two different size distributions, by intensity, and by number, as well as diameter size of nanoparticles. intensity and number distribution are presented in percentage. Settings used are exact the same as in previous measurements shown.



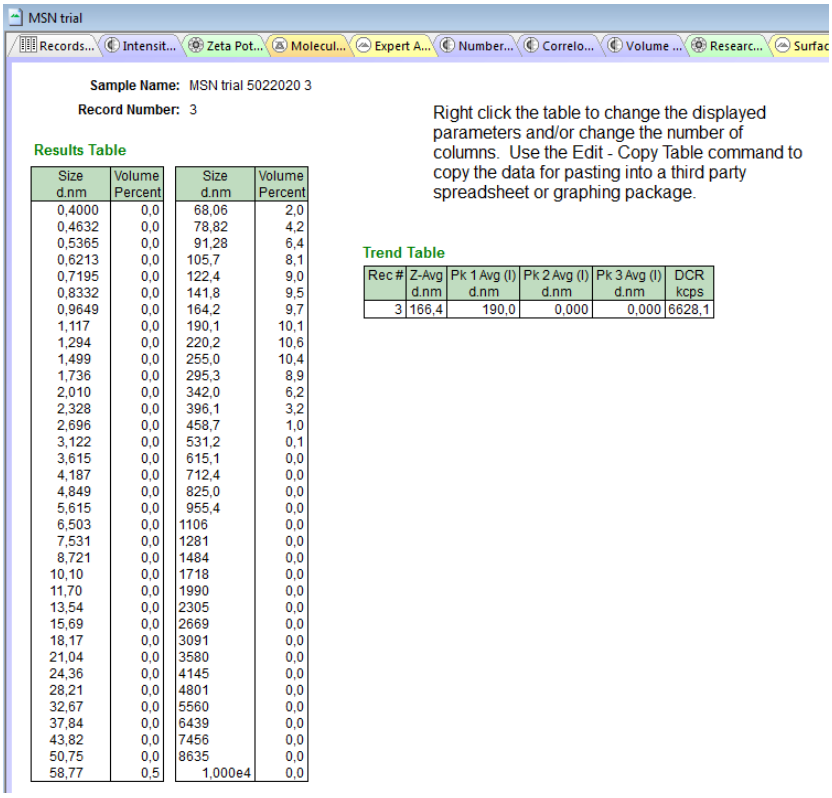


Figure 25. In the figures above, cumulative results for size by intensity are shown, as well as detailed results table. In the first figure, All of the results are shown together in the one graph, showing the size of distributed particles in nanometre diameter, along size distribution by intensity shown in percentage. Settings are used as for the previous

measures, but shown as cumulant results in this figure, including polydispersiry of the particles.

As for the results table, it shows very excessive table of measured sizes, shown as diameters in nanoscale (nanometres), and their volume percentage. This table proves very useful because it shows how much of the taken sample is consisted of the particles, and what is their size. This represents core information when it comes to the MSN synthesis, since usually these nanoparticles are within 100nm range. From this it can be seen if it was possible to synthesize nanoparticles with microfluidics, what is their size, as well as how much was produced within one sample. As this table shows, nanoparticles were created, and high percentage of them was within desired nanoscale. In our sample there were particles ranging from 78-122 nanometres in diameter, and particles with this size ranged from 2% to 9% of the volume percentage, depending on a size. It is also quite visible that a lot of particles were created within the sample, and with slightly bigger size than expected, ranging from 141-342 nanometres in diameter. Some of them were quite distributed within the sample, corresponding to around 9% of the volume percentage for various sized particles. A small percentage was consisted of very big particles with up to 531 nanometres in diameter.

The measurements taken with Zetasizer using Dynamic light scattering were proved to be of major significance. They have shown that is definitely possible to synthesize nanoparticle using microfluidic systems, and within desired nanorange, which is around 100nm in diameter. It was also noticeable that quite decent amount of created nanoparticles are above the desired nanorange, making them less useful in drug delivery. This brings very promising results, but indicates that optimisation settings can be adjusted, in order to create and synthesize particles with more constant size and distribution, which would make an ideal product for drug loading, drug delivery and use in pharmacokinetics.

5 DISCUSSION

Despite the fact that in general this is a novel method that has not been done in this manner before, it was consisted of few already established protocols. Designing and constructing the chip were mostly already established protocols, with some minor tweaks and adjustments needed, depending on a purpose of the chip. Establishing the protocol for microfluidic system, was not predetermined and was based on trial and error methods, and it had to be separated in two parts. First was just creating water phase droplets, while other part was synthesising Mesporous Silica Nanoparticles inside the droplet. This method of synthesising nanoparticles is a novel method, and did require a lot of work, adjustments and different approaches during the process. The reason behind this lies in numerous parameters that can be adjusted at the same time, and with introduction of more solutions, the number of possible combinations increases. That is the reason why the two optimisation steps were that complex, and consumed most of the time spent on this experiment. Both microfluidic optimisations were done from the outset, and were changed and redeveloped on the go, but they proved to be very promising in future work. Creating both stable droplets, and nanoparticles inside, was just and introduction for very important step, imaging. Imaging and image analysis were core steps of this experiment, because they allow converting the data to numbers, making the results of the experiment perceptible. Once the optimal flows were determined, samples were collected, and images were taken, assigning suitable image analysis protocol was needed. During the establishment, image analysis protocol also required trial and error, and different as well as more creative approaches to tackle the noise, uneven background and all the artefacts caused during imaging process. From this, it can be concluded that imaging can be done in a better way or method, using better settings than ones used in this experiment with High Speed Digital Camera Microscope. Although imaging was not perfect, it did provide images good enough that can be prone to image analysis, and all the problems faced during imaging were possible to overcome by creativity during image analysis. For example, uneven background lightning was compensated by duplicating image, adding Gaussian blur to the duplicated image, then subtracting the two images thus creating third one with more even background lightning, meaning it is more suitable for particle analysis and lowering the possibility of background to interfere with analysis itself.

When it comes to creating stable water phase droplets inside an oil phase, protocol established proved to be quite successful. A lot of samples were collected using different settings, and all of them had quite stable droplets, as it is shown in the results section. From the images of these samples, it can be seen and concluded that lower concentration oil phase is more suitable for creating droplets. Higher concentration, such as 6% created more viscous solution, making it harder to image and analyse. From this optimisation step, after the imaging and analysis, it is concluded that 2% and 4% span80 solutions provide good oil phase which has good HLB in order to create stable droplets. After analysis of each sample, it can be interpreted that physical properties of the droplet, such as size, are directly proportional to the fluid flow speeds used, and it does follow a certain trend. Few samples had bigger particle size than expected, but reason for this lies in imaging or image analysis. From the graphs it is clearly visible that higher the water phase speed is, the lower the particle size. This can be explained in a sense that higher speed creates higher force that water phase uses to exit the capillary, thus creating more particles at the time, as well as smaller in size. Results and methods from this step can be re-used in some other experiments that require creating water phase droplets inside an oil phase, but imaging technique can be improved.

When it comes to synthesis of MSNs inside a droplet, this experiment has brought a lot of negative results, with some results that look promising for future experiments. As this was a more complex, second optimisation part, and was representing the novel method, this outcome was expected. Nevertheless it did bring promising results, since not all the sample turn out to be what is considered a negative result. One sample did give stable particles, and after following all the protocol steps, DLS managed to read that there are particles formed within desired range (in nanometres). This proves that this method can be used in order to synthesize MSN particles inside a single droplet. If this protocol gets finer tuning, it is possible to create a full product that will bring promising opportunities to the field of pharmacokinetics and drug delivery fields. Negative results also don't necessarily bring bad news but provide good source of information as well. Negative results obtained during this experiment can be used as a guideline and starting point for similar experiment, or recreation of this one. The fact that it was within reach to synthesize nanoscale particles inside a microfluidic chip proves that hypothesis of this experiment was correct and it is possible and very imaginable to do it to full extent. Proof for this lies in data acquired from DLS, which clearly indicates particles within nanoscale synthesised inside a droplets that are within sample

The final step for the whole process, and a step that would be crucial for validating all the results obtained, is Transmission Electron Microscopy imaging, which would be able to image the nanoparticles, as well as their physical properties, such as shape, size and topography. Unfortunately, during this experiment, and prior to this crucial and final step, outbreak of the COVID-19 virus has taken its toll by shutting down the labs and imaging facilities, so it was impossible to finish this experiment and get final validation of the fact if the created particles were nanoparticles indeed or not. Since it was not possible to go through with TEM, results from DLS could not be confirmed with images, and it cannot be concluded with one hundred percent rate that the particles synthesized and analysed were mesoporous silica nanoparticles. Nevertheless, with all the aforementioned problems faced, which were expected since it is a novel method, it can be said that this new way of using microfluidic systems in order to produce MSNs is definitely possible, and it has great potential to find its place in a fields of drug delivery, drug loading and pharmacokinetics.

What can be concluded is that the premise of this experiment stands on solid ground, but execution can be improved, especially in terms of imaging techniques. In order to finalise this experiment to its fully potential, it is necessary to improve mixing of the TEOS and CTAB within the microchip, and imaging as it is of core significance. Better imaging of droplets within the sample makes an easier image analysis, thus more solid results, and this can be done by using better microscope to image the sample after collecting, while High Speed Digital Camera Microscope should be kept as a monitoring tool.

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