Nanoemulsion Based on Renewable Polymer for Improved Delivery of Therapeutics

Master's thesis by

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2019

Abstract

Poor water solubility is a common problem with new drug candidates. Water solubility of a drug has an important role in the absorption and will consequently affect the bioavailability. One approach to enhance the solubility of poorly soluble drugs is to create nanoemulsions. A nanoemulsion is the dispersion of one immiscible liquid into another immiscible liquid, and their small droplet size gives unique properties beneficial to the drug delivery systems. The immiscible liquids in a nanoemulsion are usually oil and water. When using natural oils, they tend to go rancid or cause Ostwald ripening to the emulsion. Renewable poly(δ-decalactone) (PDL), being an oily polymer, could be used to replace oil in preparation of nanoemulsions. Therefore, the PDL polymers' ability to be made into nanoemulsions and load drugs was studied.

The intention of this study was to show a simple way of preparing nanoemulsions from polymers. Five hydrophobic drugs were loaded into the nanoemulsions. The nanoemulsions were prepared with a nanoprecipitation method, by dropwise adding the polymeric oil phase to the water phase under stirring. The nanoemulsions were characterised in terms of size, drug loading, stability, and cell toxicity.

Results from the study show that nanoemulsion with droplet size less than 200 nm was achieved using the PDL polymer. This emulsion was also more effective than Pluronic F-68 micelles alone in terms of drug encapsulation. The nanoemulsions were proven to be stable over a storing time of three months, at two different storing temperatures (20 °C and 50 °C). Regarding cell toxicity, both the nanoemulsions and the Pluronic F-68 micelles demonstrated a time and concentration-dependent toxicity; however, no cytotoxicity was induced by the PDL polymer itself. The study also shows that the PDL polymer has the ability to serve as the oil phase in drug delivery applications.

Keywords: Nanoemulsion, Polymeric drug carrier, Particle size, Stability

Contents

1 Introduction

1.1 Solubility issues in drug development

It is well known that solubility, dissolution, and gastrointestinal permeability are essential parameters that control the rate and extent of drug absorption and eventually bioavailability. The water solubility of drugs plays a significant role in the formulation (such as tablets, capsules, syrups, and injections) and absorption of drugs in the human body. In drug discovery, the number of insoluble drug candidates has increased in recent years, with almost 70% of new drug candidates showing poor water solubility. For these drug candidates, poor aqueous solubility and poor dissolution in the gastrointestinal (GI) fluids is a limiting factor to the *in vivo* bioavailability after oral administration (Khadka, et al., 2014). Due to low formulation costs and superior patient compliances, administration of drugs via oral route is preferred and, therefore, the drug must be dissolved in the GI fluids to achieve sufficient absorption. When a drug is absorbed through the GI tract, it reaches the systemic circulation via the blood stream to hit the target site (Maleki, et al., 2017). Drugs with poor solubility are usually administered in high doses to achieve sufficient pharmacological response, but this approach increases the risk of toxicity. In addition, formulating therapeutics with a high drug-loading could be difficult and apparently enhance the manufacturing costs and, therefore escalating the dose to achieve optimum therapeutic response is often an undesirable method (Lu & Park, 2013). Therefore, increasing the dissolution rate of poorly soluble drugs is an important challenge to pharmaceutical scientists (Khadka, et al., 2014).

Solubility (or dissolution rate) can be enhanced by physical, chemical, or other modifications on the drug molecules. Several techniques, such as pH modification, salt forms, emulsification, co-solvency, surfactant solubilisation, size reduction, or employing nano-carriers (for e.g. polymeric micelles, liposomes, nanoparticles) are routinely utilised to enhance the aqueous solubility and bioavailability of poorly soluble drugs (Kalepu & Nekkanti, 2015; Maleki, et al., 2017).

In this thesis, nanoemulsions, and their ability to enhance bioavailability of poorly water soluble drugs, are studied.

1.2 Nanopharmaceuticals as solubility enhancers

Drug loading and encapsulation efficiency depend on drug solubility in the excipient matrix material. The amount of matrix materials used for administration can be reduced, if the drug-loading capacity of the nanodelivery system is high. An important characterisation of the nanoparticles is naturally the particle size and particle size distribution, since they will determine aspects such as targeting ability and biological fate. It has been reported that nanoparticles can be formulated to cross several biological barriers, including the blood-brain barrier. (Singh & Lillard Jr, 2009).

Most of the nanopharmaceuticals that have already entered the market are previously existing drugs conjugated to nanoparticles to improve the pharmacokinetic, or the pharmacodynamic, properties of the drug molecules. Typical benefits of the existing nanopharmaceuticals are greater protein stability, decreased systemic toxicity, and longer circulation time compared to the conventional drugs. In clinical trials anticancer and antimicrobial nanodrugs are currently the drug classes where most formulations are being developed to improve their safety and efficacy. Formulations for other indications, such as autoimmune conditions, anaesthesia, metabolic disorders, ophthalmic conditions, neurological and psychiatric diseases are also under development. (Ventola, 2017).

When developing new nanopharmaceuticals it is important to study the adverse effects of the product. Nanotoxicology is the safety assessment of engineered nanomaterials for medical application. Even if one of the goals with nanopharmaceuticals is to improve the safety and reduce the toxicity, there are still potential risks with the nanomaterials themselves. Concerns have been raised about how the nanoparticles might affect the human health and the environment in an undesirable way. Nanoparticles could, for instance, undesirably enter the blood circulation when administered through the airways or accumulate in the body. The idea of nanotoxicology is to identify the potential hazards as well as to do safety evaluations of nanopharmaceuticals. Toxicological studies should also be carried out using realistic doses both *in vitro* and *in vivo* (Oberdörster, 2010).

1.3 Nanoparticles as drug delivery systems

Nanomedicine is a growing field that combines nanotechnology with pharmaceutical and biomedical sciences. The term nanomedicine includes nanopharmaceuticals, nanoimaging agents, and theranostics (therapeutic and diagnostic) (Ventola, 2017). Nanomedicines are nanoparticles that incorporate drugs or biologics in order to attain improved targeting, improved solubility and pharmacokinetics, reduced toxicity, improved safety or otherwise enhanced efficacy (Bobo, Robinson, Islam, Thurecht, & Corrie, 2016).

Nanomedicines can be divided into three major categories, i.e. lipid based (liposomes), polymeric based (polymeric micelles), and inorganic based (gold nanoparticles) depending on the material used to prepare them (Figure 1). The use of single polymer chains is the most basic class of polymeric nanomedicines, directly as the therapeutic or as a modifying agent for a drug. Poly (ethylene glycol) is the most popular polymer and is utilized, for instance, in the drug Neulasta®, used for chemotherapy-induced neutropenia. Polymeric micelles are made up of self-assembled polymeric amphiphiles designed for controlled delivery of hydrophobic drugs (Bobo, Robinson, Islam, Thurecht, & Corrie, 2016). Today, there are few authority-approved polymeric micellar formulations, but Genexol®PM is a product that is already on the market. Genexol®PM is a polymeric nanoparticle micelle formulation of paclitaxel that is used for the treatment of breast cancer and non-small cell lung cancer (Werner, et al., 2013). Several micellar formulations are also in clinical trials, including intravenously administered micellar systems. The most easily synthesized type of nanoparticles are liposomes. They are self-assembling and the system enables delivery of hydrophobic and hydrophilic compounds. Classical liposomes typically exhibit short circulating half-lives due to fast clearance, in terms of intravenous delivery. (Bobo, Robinson, Islam, Thurecht, & Corrie, 2016).

Another type of nanomedicines is protein-based nanoparticles which are drugs conjugated to protein carriers, complex combined platforms that use proteins for targeted delivery, or drugs were the protein itself is the active therapeutic. Protein-based nanoparticles earlier exploited the properties of proteins in blood serum, so the drugs were transported and dissolved during circulation. Dendrimers are composed of three distinct architectural regions arranged concentrically around a central core. This configuration allows drug substances to be encased within the cavity around the core or to be connected to the nanoparticle surface. Nanomedicines

have successfully been made with dendrimers administered intravenously and orally, among others. (Ventola, 2017).

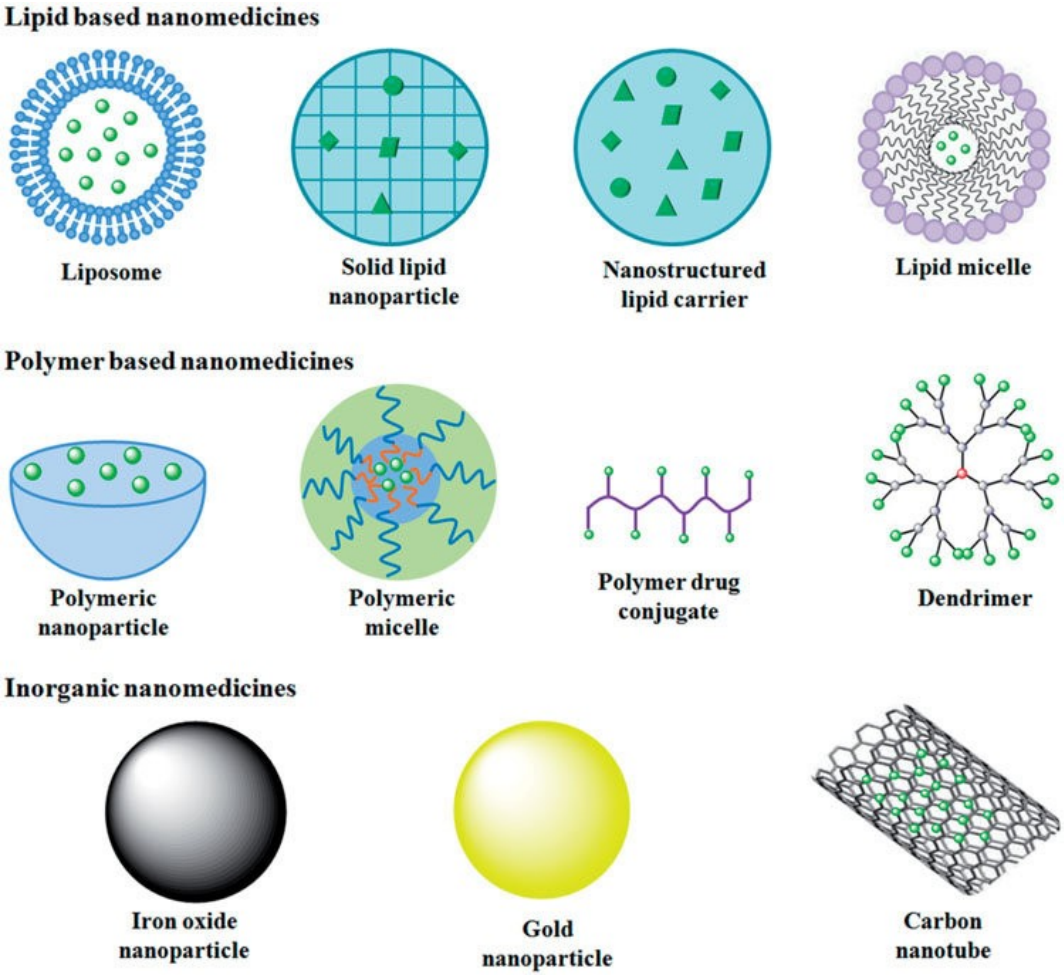

Figure 1. Different types of nanocarriers (Rizwanullah, Amin, Mir, Fakhri, & Rizvi, 2018).

Nanoparticles can also be made with metallic and metal oxide materials, such as gold or iron oxide. In clinical trials, gold has been used as a nanomedicine and a therapeutic with iron oxide nanoparticles has already been regulatory approved. The nanoparticles in crystalline nanomedicines consist only of the drug compound and methods such as milling and homogenization are used for the synthesis. Both organic drugs and inorganic materials can be made into crystalline nanoparticles. However, categorising nanoparticles by materials is not always straight-forward, since new nanomaterials often contain elements of a number of different materials, and micelles and liposomes are now often synthesized using synthetic polymers. Furthermore, instead of relatively simple nanoparticles, complex multi-layered particles, such as

silica nanoparticles or hydrogels, have grown in popularity. (Bobo, Robinson, Islam, Thurecht, & Corrie, 2016).

1.4 Complex particles

Modern approaches in protein engineering, and advances in polymer and inorganic chemistry has resulted in an expansion of novel nanomaterials, that blur the boundaries between the categories of traditional materials. This gives systems with more complex functions and more complex structures (Ventola, 2017). Increased complexity can also lead to higher costs, and scale-up can be challenging. Complex multi-layered particles can be created from silica nanoparticles, metal oxide nanoparticles, nanotubes, dendrimers, polymers, cyclodextrins, lipids, hydrogels, and semiconductor nanocrystals (Bobo, Robinson, Islam, Thurecht, & Corrie, 2016).

The mesoporous silica materials are characterized by uniform pore size, large surface areas, significant biocompatibility, and easy surface functionalization. They are usually prepared by hydrolysis and condensation of silica precursors around the micelle templates. The micelle templates are later removed from the silica material. The mesoporous silica materials can be synthesized to different sizes and different pore diameters as well as to different type of morphologies. (Maleki, et al., 2017).

With a nanosuspension as the ink, nanodrugs have also been inkjet printed onto edible porous substrates. A nanosuspension could possibly enhance the bioavailability of poorly soluble drugs, and with this method the drugs can be administered in personalized dosage forms. The printed dose could be increased, towards a therapeutic dose, by using a drug nanoparticle suspension as the ink medium, instead of a conventional drug solution. (Cheow, Kiew, & Hadinoto, 2015).

Multifunctional nanoparticles can act as targeted nanomedicine for cancer treatment. Thanks to their unique physical and chemical properties, multifunctional nanoparticles can cross cancer cell barriers and deliver chemotherapeutic agents to the target site. The multifunctional nanoparticles can consist of lipid-based, polymer-based, and inorganic nanoparticles. These types of novel nanomedicines are promising and would be beneficial for

the patients; however, more research is required on the subject of complex drug delivery systems. (Rizwanullah, Amin, Mir, Fakhri, & Rizvi, 2018).

1.5 Nanoemulsions as drug delivery systems

Nanoemulsions are defined as small droplets of one immiscible liquid dispersed in another immiscible liquid. The liquids are usually oil and water (McClements & Jafari, 2018). Nanoemulsions are used for targeted drug delivery of several anticancer drugs, photo sensitizers or therapeutic agents, and they can also prolong the action of the drugs. There are numerous advantages of using nanoemulsions, such as the large surface area which will facilitate the absorption. Nanoemulsions are also non-toxic and non-irritant in nature, and thanks to their smaller droplet size, they have better physical stability than conventional emulsions (Jaiswal, Dudhe, & Sharma, 2015). The therapeutic efficacy of the drug is enhanced, while adverse effect and toxic reactions are minimized. Nanoemulsions can be formulated into several dosage forms, such as liquids, creams, gels, sprays, aerosols, and foams. The administration routes for nanoemulsions can vary accordingly, including oral, intravenous, pulmonary, transdermal, intranasal, and ocular routes. (Singh, et al., 2017).

There is some disagreement on which droplet size defines a nanoemulsion. Some sources (Odriozola-Serrano, Oms-Oliu, & Martín-Belloso, 2014) suggest that the particles should be between 10 and 100 nm, whereas others states that the droplets can be between 50 and 1000 nm (Lovelyn & Attama, 2011). McClements and Jafari (2018) propose a droplet size below 200 nm as a border to differ nanoemulsions from conventional emulsions. Since nanoemulsions below 200 nm tend to have more bioavailability, higher stability, higher apparent transparency, and more chemical reactivity. There can be oil-in-water and water-in-oil forms of nanoemulsions, with oil-in-water being more common. Using nanoemulsions as building blocks, multiple emulsions (oil-in-water-in-oil or water-in-oil-in-water) or other structures can also be produced (McClements & Jafari, 2018). In addition to water and oil, as an essential component, an emulsion also needs an emulsifier. The emulsifier is usually a surfactant (e.g. Tween or Pluronic), but proteins or lipids can also be used. Some nanoemulsions can also contain preservatives, antioxidants or chemoprotectants (Singh, et al., 2017). Nanoemulsions are easily produced in large quantities by mixing a water-

immiscible oil phase with an aqueous phase under high shear stress or with a mechanical extrusion process (Lovelyn & Attama, 2011). The applicability of nanoemulsions is not limited to drug delivery. In the food industry, flavoured nanoemulsions have been prepared with improved curcumin/β-carotene digestibility. Research has shown that many problems in current methods of pharmaceutical crystallization processes can be avoided with nanoemulsions (Gupta, Eral, Hatton, & Doyle, 2016). The nanoemulsions are relatively stable due to the small droplet size; and thus, no creaming or sedimentation occurs during storage. The large surface area of the emulsion system allows rapid penetration of therapeutics, and they are therefore suitable for efficient delivery of active ingredients through the skin (Lovelyn & Attama, 2011).

As mentioned earlier, nanoemulsions usually consist of an oil phase and an aqueous phase. The oil phase can consist of different hydrophobic components, such as triglyceride oils, essential oils, flavour oils, oil-soluble vitamins, colours, flavours, preservatives, nutraceuticals, and pharmaceuticals. The aqueous phase mainly consists of water, but components, such as buffers, salts, cosolvents, preservatives, proteins, and carbohydrates can be included in the aqueous phase. Therefore, the physicochemical and physiological properties of a nanoemulsion is not only dependent on the oil phase, but also the aqueous phase. (McClements & Jafari, 2018).

Preparation methods of the nanoemulsions can be divided into two main groups; high-energy, and low-energy emulsification. Typically, nanoemulsions are prepared in a way where first macroemulsions are formed and then converted into nanoemulsions (Gupta et al., 2016). The high-energy emulsification methods include high-energy stirring, ultrasonic emulsification, high-pressure homogenization, and microfluidization. The low-energy emulsification methods include phase inversion temperature, emulsion inversion point, and spontaneous emulsification. (Jaiswal, Dudhe, & Sharma, 2015). Membrane emulsification is sometimes categorised as a high-energy emulsification method, but the mechanical technique does not use a lot of energy. An emulsification method is chosen according to the properties of the materials that are used, and the desired form of the final product. Therefore, the particles in a nanoemulsion vary in terms of size, electrical charge, and physical state (Figure 2). The properties of the particles in a nanoemulsion can be tuned to achieve the required physicochemical or physiological properties. (McClements & Jafari, 2018).

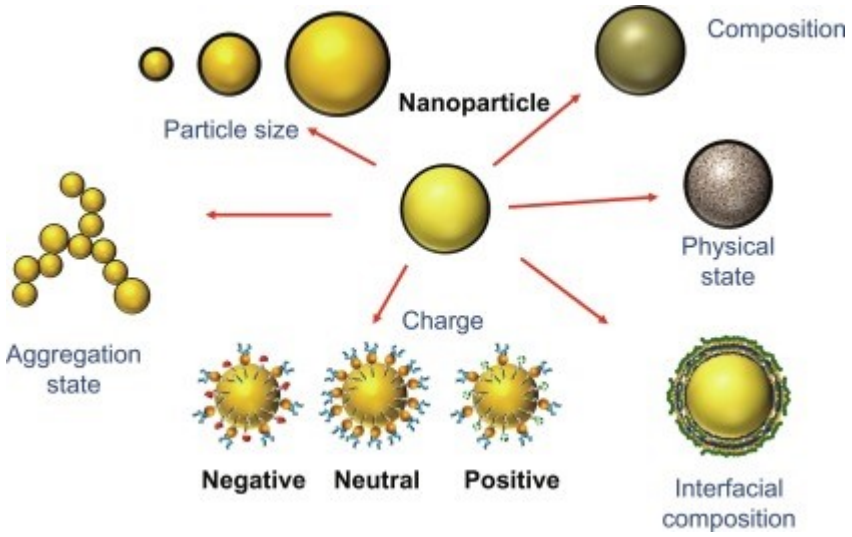

Figure 2. Different particle characteristics of nanoemulsions. (McClements & Jafari, 2018)

1.6 Challenges with nanoemulsions

Despite several advantages with nanoemulsions, there are a few disadvantages associated with this system, which should be considered when choosing it as a drug delivery vehicle. A limiting factor in development of nanoemulsions is the requirement that all components should be strictly non-toxic. High manufacturing cost due to expensive instruments is also one of the factors restraining the use of this system. (Singh, et al., 2017).

Ostwald ripening is the major destabilization mechanism of nanoemulsions. It is a process where larger droplets grow on the expense of smaller droplets in the emulsion. Ostwald ripening is driven by the Kelvin effect. Due to difference in Laplace pressure, the small emulsion droplets have higher local oil solubility than the larger droplets. Molecular dissolution of oil in the continuous phase is the main way through which Ostwald ripening occurs. (Wooster, Golding, & Sanguansri, 2008).

It has been suggested that the Oswald ripening process could be prevented by addition of a hydrophobic component into the oil, which significantly decreases the coalescence rate, thus producing a kinetically stabilized nanoemulsion. The use of polymeric stabilizers has also been reported to slow down the Ostwald ripening for shorter periods of time (between 24 and 48 hours) (Chebil, Desbrières, Nouvel, Six, & Durand, 2013). In addition, the droplets in the nanoemulsions

prepared using a high viscosity oil, such as long chain triglycerides (LCT), were larger than the droplets in the nanoemulsion made using a low viscosity oil, such as hexadecane. The nanoemulsions made with LCT did not experience Ostwald ripening, and were physically stable for more than three months. The large molar volume of LCT oils makes them insoluble in water, which creates a kinetic barrier that prevents Ostwald ripening. It has been reported that nanoemulsions can be thermodynamically stable to Ostwald ripening, if at least 50% of the oil phase consists of an insoluble triglyceride. (Wooster, Golding, & Sanguansri, 2008).

Rancidity is a problem with vegetable oils. Enzymatic rancidity is caused by the presence of certain enzymes. Time, temperature, light, air, and exposed surfaces are factors that cause oxidative rancidity (Riaz & Rokey, 2012). Oxidation is the main cause for deterioration of quality for oils and fats. Oxidation of lipids occurs when unsaturated fatty acids react with oxygen. When oxygen from the atmosphere is added to the fatty acids, unpleasant flavour, aroma compounds, or discolouration will eventually form. Oxidation can, in addition to make the oil unpleasant, also produce toxic compounds. In the food industry, removal of oxygen, gas barrier packing materials, and addition of antioxidants are approaches used for avoiding oxidation (Akoh & Min, 2002).

Even though nanoemulsions can be stable for years, there is always the possibility of break down over time, due to gravitational separation, flocculation, coalescence, and Ostwald ripening. Because of this stability problem, nanoemulsions often need to be prepared relatively shortly before use, and a large quantity of surfactants and cosurfactants are often needed. Also, the knowledge of what role surfactants and cosurfactants have in production of nanoemulsions is still deficient. (Chime, Kenechukwu, & Attama, 2014). A phase diagram is constructed, to study all the types of formulations that can occur when making a nanoemulsion. To prepare a phase diagram, different ratios of water, oil, and surfactants are mixed. The results provide information about which concentrations of the different components should be used when preparing a nanoemulsion. (Mat Hadzir, et al., 2013).

1.7 Applications of nanoemulsions

A lot research on nanoemulsions has been carried out in the pharmaceutical field. A study made on a nanoemulsion formulation of ramipril used Sefsol 218 as the oil phase, and triacetin, IPM, Labrafac, and castor oil as the other compounds. The nanoemulsion was created using a vortex, and the study showed that it could be used as a drug delivery system. (Shafiq, et al., 2007). Cyclosporin A is currently on the market as a microemulsion administered parenterally to the eye. Lipid-based adjuvants, formulated as nanoemulsions, are approved for use in human vaccines; a squalene-based adjuvant is used as a component of an influenza vaccine (Peshkovsky & Bystryak, 2014). Curcumin has been undergoing clinical trials as a nanoemulsion, targeted for atypical ductal breast hyperplasia (Singh, et al., 2017).

1.8 Polymers used in the fabrication of nanoemulsions

Recently, poly(decalactone) (PDL) polymer was reported as a viscous oil type material, synthesised using a renewable monomer (Bansal, et al., 2015). The copolymer prepared, using PDL, was found to be biodegradable, and less toxic *in vitro* and *in vivo*. The nanoemulsion of PDL could be readily obtained, simply by nanoprecipitation method, thus avoiding use of sophisticated instruments in the preparation phase. It was anticipated, that as an oily polymer with high hydrophobicity and viscosity, PDL could be able to make a stable nanoemulsion resistant to Ostwald ripening and rancidity, which is often observed with natural oils.

As previously mentioned, there are several approaches to make nanoemulsions, but they are often difficult or expensive. Preparing a phase diagram is a primary requirement to obtain a stable emulsion. A phase diagram is needed to determine the optimal concentration of oil, surfactant, and cosurfactant, which can generate a nanoemulsion with good stability (Figure 3). However, with polymeric nanoemulsions, a stable system with small droplet size can be produced without the use of a phase diagram.

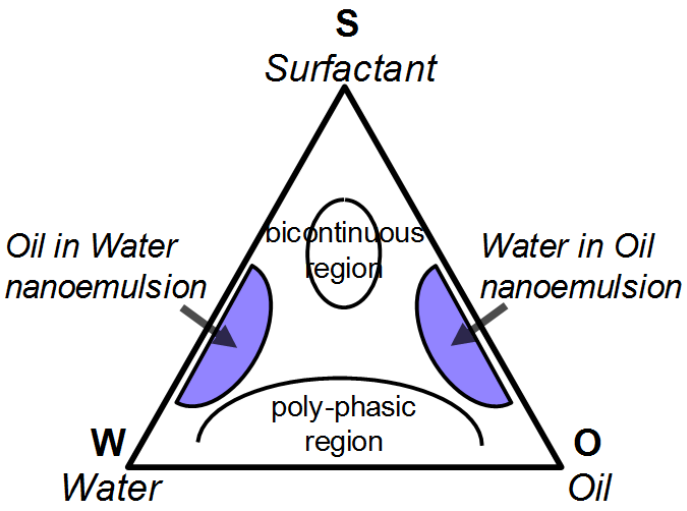

Figure 3. Schematic phase diagram, the shaded areas are the regions were nanoemulsions are found (Yang & Xu, 2016).

Pluronic F-68, also known as Poloxamer 188, or poly(ethylene oxide) (PEO) –b-poly- (propylene oxide) (PPO) –b- poly(ethylene oxide) (PEO), block copolymer was chosen as a surfactant to stabilize the nanoemulsion due to its well-known stabilization property and FDA approval for human use. This triblock copolymer has already been used in several studies to create a stable nanoemulsion (Wulff-Pérez, Gálvez-Ruíz, De Vicente, & Martín-Rodríguez, 2010; Wulff-Pérez, Torcello-Gómez, Gálvez-Ruíz, & Martín-Rodríguez, 2009). Pluronic block copolymers have the ability to self-assemble into micelles in aqueous solutions, when the concentration of the copolymers goes above the critical micelle concentration.

1.9 Drug molecules investigated in this study

Hydrophobic drugs, such as carvedilol, curcumin, cyclosporin A, griseofulvin, and prednisolone, were chosen in this study. The selected drugs differ in pharmacological action, intrinsic aqueous solubility, and molar mass, and could therefore provide a better indication of the large applicability of a nanoemulsion as a drug carrier. Carvedilol is used as an antihypertensive medication, it acts as a non-cardioselective beta blocker and vasodilator, and is practically insoluble in water (National Center for Biotechnology Information, n.d.a). Curcumin is obtained from turmeric, and possesses a variety of pharmacological properties, mainly because of its inhibitory effect on metabolic enzymes. It is an orange-yellow crystalline powder that is insoluble in water (National Center for Biotechnology Information, n.d.b). Cyclosporin A is an

immunosuppressive agent that is only slightly soluble in water. It is currently on the market under the name Sandimmun® and is used after organ transplantation, or in the treatment of autoimmune disease (National Center for Biotechnology Information, n.d.c). The solubility of griseofulvin is less than 10 mg/l in water. It is an antifungal agent that is used to treat fungal skin and nail infections (National Center for Biotechnology Information, n.d.d). The solubility of prednisolone is 223 mg/l in water at 25 °C, which makes prednisolone the most water-soluble drug among all drugs used in the study. Prednisolone is a synthetic glucocorticoid, with anti-inflammatory and immunosuppressive properties (National Center for Biotechnology Information, n.d.e).

2 Aims

The aim of this study is to develop and characterize, a stable polymeric nanoemulsion system via facile methodology for drug delivery applications.

3 Materials and methods

3.1 Materials

The polymer used in this study was synthesised using a renewable monomer δ-decalactone. δ-Decalactone is an FDA-approved flavouring agent and a candidate monomer for biomedical polymer applications. The drugs cyclosporin A (Sigma-Aldrich, USA), carvedilol (Sigma-Aldrich, USA), curcumin (Sigma-Aldrich, China), prednisolone (Sigma-Aldrich), and griseofulvin (Fagron) were used as received. All the solvents used were purchased from Fischer Scientific UK. Pluronic F-68 (10%,) and triton X-100 (BioXtra) were also purchased from Sigma-Aldrich. Milli-Q® water was used for preparing aqueous solutions.

3.2 Methods

3.2.1 Size exclusion chromatography

Size exclusion chromatography (Shimadzu, Germany) was used to determine the number-average molar mass (Mn), weight average molar mass (Mw), and mass distribution (polydispersity (PD), Mw/Mn) of the polymer. Tetrahydrofuran (THF) was used as mobile phase at 40 °C with a flow rate of 1 ml min^{-1}. The instrument was fitted with a low temperature evaporative light scattering detector (LT-ELSD) with AM GEL linear column and AM gel guard column (300 x 7.8mm). Column calibration was done using narrow polystyrene standards of known Mn and PD in the range of 600 Da– 2300kDa. The chemical structure of the polymer was examined by proton nuclear magnetic resonance (1H-NMR) spectroscopy, using a Bruker NMR 500 MHz spectrometer (Bruker, Coventry, United Kingdom). Deuterated chloroform (CDCl3) was used as a solvent.

3.2.2 Viscosity

The viscosity of the polymer was determined using a TA instrument rheometer (AR 2000). Measurements were done at 23° C, at shear rates ranging from 1–45 s-1. The viscosity was determined using rheology data advantage analysis software version 7.0 by fitting the data using viscosity vs rate.

3.2.3 Synthesis of poly(δ-decalactone)

The poly(δ-decalactone) (PDL) polymer was synthesised according to a reported procedure via ring opening polymerisation (ROP) of monomer δ-decalactone in the absence of solvent (Bansal et al., 2015). Monomer (δ-decalactone, 10.00 g, 58.7 mmol) was added to a flask containing initiator (propargyl alcohol, 0.03 g, 0.6 mmol) and stirred well to make a homogeneous mixture. Catalyst (TBD, 0.20 g, 1.4 mmol) was then added to the flask, and the final mixture was allowed to react for eight hours at room temperature. The obtained viscous liquid was later quenched by adding benzoic acid (0.35 g, 2.9 mmol) solution in acetone, precipitated in cold methanol (twice) and the residual solvent was evaporated under vacuum. The polymer propargyl-PDL was recovered as colourless viscous liquids with a yield of 8.03 g (80%).

^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) 4.86 (CH2-C<u>H</u>–O–C=O, m, 102H), 4.66 (C-C<u>H_2</u>–O-CO, d, 2H), 3.69 – 3.45 (CH_2–C<u>H</u>–O<u>H</u>, m, 4H), 2.46 (C≡C<u>H</u>, s, 1H), 2.44 – 2.12 (O–CO–C<u>H_2</u>, m, 213H), 2.44 – 2.12 (C<u>H_2</u>–C<u>H_2</u>–CH– C<u>H_2</u>, m, 616H), 1.35 – 1.05 (C<u>H_2</u>–C<u>H_2</u>–C<u>H_2</u>–CH_3, m, 631H), 0.86 (CH_2–C<u>H_3</u>, t, 305H).

^{13}C NMR (126 MHz, $CDCl_3$) δ (ppm) 173.50, 173.08 (CH–O–<u>C</u>O, CH_2–O–<u>C</u>O), 74.82 (<u>C</u>H–C–CH_2), 73.69, 71.34 (CH_2–<u>C</u>H–O–CO, CH_2–<u>C</u>H–OH), 73.55 (CH–<u>C</u>–CH_2), 51.84 (CH–C–<u>C</u>H_2), 37.48, 34.20 (CH–<u>C</u>H_2–CH_2), 36.84, 33.48 (<u>C</u>H_2–CH–OH, <u>C</u>H_2–CH–O–CO), 33.96, 34.45 (O–CO–<u>C</u>H_2), 31.89, 31.66 (<u>C</u>H_2–CH_2–CH_3), 25.35, 24.96 (CH–CH_2–<u>C</u>H_2), 22.65, 22.54 (O–CO–CH_2– <u>C</u>H_2), 20.80 (<u>C</u>H_2–CH_3), 14.01 (CH2–<u>C</u>H_3).

Theoretical molecular weight (MW) – 15.3 kDa,

Calculated MW by ^{1}HNMR: 17.4 kDa

MW by SEC: Mn – 9.4 kDa, Mw – 11.4 kDa, Mz – 14.4 kDa, PD – 1.21

Viscosity: 62.20 Pa.s

3.3 Preparation methods of nanoemulsions

A nanoprecipitation method was used to prepare nanoemulsion using PDL polymer as oil and Pluronic F-68 as surfactant. Drug loaded oil-in-water nanoemulsions were prepared by dissolving drug (5 mg) and polymer (PDL 25 mg) in solvent (acetone 1.5 ml). This organic mixture was then added dropwise to the Milli-Q water (3.5 ml), containing surfactant (Pluronic F-68, 1.5 ml) with stirring (1000 rpm). The solution was then stirred for at least three hours at room temperature and left overnight (open vial) to ensure the complete removal of organic solvent. The nanoemulsion was finally filtered through a membrane syringe filter (pore size: 0.45 μm) and used for further characterisation. Simultaneously, a similar mixture was prepared for comparison purposes using Pluronic F-68 only. A blank nanoemulsion was prepared following a similar process but without drug. Mixtures containing only drug, solvent, and water were also prepared, but these were not used for the final characterisations.

Five drugs were used for the preparation of drug loaded nanoemulsions, i.e. cyclosporin A, carvedilol, curcumin, prednisolone, and griseofulvin. Curcumin is light sensitive, and the preparation of the curcumin loaded nanoemulsion was performed under dark.

3.4 Nanoemulsion characterization

3.4.1 Estimation; Preparation of standard curves, UV and HPLC

Ultraviolet-visible (UV-Vis) spectra were obtained using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA). Collection of UV spectra was carried out, using either a capped quartz cuvette with a sample volume of 1000 µl, or the drop technique, after suitable dilutions. The amount of drug present in the samples was calculated using standard calibration curves prepared with a mixture of methanol and water. Drug Content (DC) and Encapsulation Efficiency (EE) were also determined from the calibration curve, using the formulae below (Bansal, Gupta, Rosling, & Rosenholm, 2018):

$$\text{DC wt\%} = \frac{\text{Weight of loaded drug}}{\text{Weight of polymer used}} \times 100$$

$$\text{EE\%} = \frac{\text{Weight of loaded drug}}{\text{Weight of drug in feed}} \times 100$$

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Concentrations of cyclosporin A was estimated using HPLC. The mobile phase used was water and acetonitrile (20:80%) with 0.05% of TFA in the acetonitrile. The temperature of column (Gemini-NX 3u C18 110A, 100 x 4.6 mm) was set to 50 °C in column. The flow rate was set to 0.7 ml/min and absorbance was measured at 210 nm (Aljohani, et al., 2017). The analysis was performed using a Merck Interface D-7000 Diode Array Detector. A standard calibration curve was made using 100 to 1000 µg per ml of cyclosporin A diluted with a mixture of water and ethanol. Samples were run for seven minutes to determine the retention time of cyclosporin A.

3.4.2 Particle size analysis (DLS)

The size and polydispersity index of globules of nanoemulsions was measured on a ZetaSizer NanoZS® (Malvern Instruments, Worcestershire, UK). Three drug samples and a blank nanoemulsion were chosen for the particle size analysis. The samples were diluted with Milli-Q water and transferred into cuvettes. Measurements were performed at 25 °C. Data analysis was carried out using the Malvern ZetaSizer software version 7.12.

3.4.3 Surface charge

The surface charge was measured using Malvern ZetaSizer NanoZS® (Malvern Instruments, Worcestershire, UK) to obtain zeta potential values. Samples were diluted with Milli-Q water before measurement. The samples were then inserted into a folded capillary zeta cell for charge measurements.

3.4.4 Transmission electron microscopy (TEM)

TEM images were taken to confirm the size and to determine the surface morphology. Samples were imaged on TEM grids without staining. TEM images were taken for both empty and drug loaded nanoemulsions, using a JEM 1400-Plus (JEOL Ltd., Tokyo, Japan). To perform the TEM observations, the nanoemulsion formulation was diluted with water and filtered through a membrane syringe filter. A drop of the diluted nanoemulsion was then directly deposited on the copper grid and observed in TEM after drying.

3.4.5 Stability study

The stability of nanoemulsions in terms of phase separation, change in size, charge, and drug degradation was evaluated by high-speed centrifugation and storage at different temperatures. Nanoemulsions loaded with prednisolone and cyclosporin A were selected for this study. Empty and drug loaded nanoemulsions were centrifuged for 30 minutes at 10000 rpm, and the phase separation was analysed visually. For long-term stability studies, nanoemulsions were stored at room temperature (20 ± 2 °C) and incubated at 50 ± 2 °C for three months. The size, zeta potential, and drug content were checked at the beginning and then after every 30 days.

3.4.6 Haemolysis

The haemolytic study was performed, according to reported methodology, with minor modifications (Evans, et al., 2013). To prevent coagulation, 5 ml of human blood, from an anonymous donor, was drawn directly into a Na2-EDTA-coated tube. The blood was then centrifuged at 500 g for 5 minutes to separate the red blood cells (RBCs) from the plasma, which was discarded. RBCs was washed twice with 150 mM NaCl-solution followed by one wash with phosphate buffer saline (PBS, pH – 7.4). RBCs were diluted up to 5 times with PBS (pH – 7.4) to make a stock suspension.

Nanoemulsions (50 mg/ml) prepared in PBS were further diluted to make 25, 12.5, and 1.25 mg/ml concentration in PBS. For each assay, 800 µl of the nanoemulsions were added to 200 µl RBCs (from stock) to make 1 ml. Therefore, the stocks of 50, 25, 12.5, and 1.25 mg/ml, resulted in concentrations of 40, 20, 10, and 1 mg/ml of nanoemulsion. Positive control tubes were prepared by adding 800 µl of 1.25% solution of triton X-100 in 200 µl RBCs. Negative control tubes were prepared by adding 800 µl of PBS in 200 µl RBCs. The tubes were incubated at 37 °C for 1 h and 24 h. After incubation the tubes were carefully handled and centrifuged at 500 g for 5 minutes. From each tube, the supernatant was then analysed on UV–Vis spectroscopy to measure the absorbance of released haemoglobin at λmax – 542 nm. From the results of the UV-Vis spectroscopy, the percentage of haemolysis was calculated using the formula below (Bansal, Gupta, Rosling, & Rosenholm, 2018):

$$\% \text{ Haemolysis} = \frac{\text{Abs of sample} - \text{Abs of negative control}}{\text{Abs of positive control} - \text{Abs of negative control}}$$

.

3.4.7 Cell study

MDA-MB-231 cells (human breast adenocarcinoma) were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 1% penicillin–streptomycin (v/v).

The cytotoxicity of the nanoemulsion was evaluated using WST-1 cell viability assay (Roche Diagnostics, Mannheim, Germany) on MDA-MB-231 cells, using reported procedure, with minor modifications (Prabhakar, et al., 2016). Briefly, 100 µl of cell stock suspension with a concentration of 50,000 cells/ml was seeded into a 96-well plate and incubated for 24 hours. The different concentrations of nanoemulsion, i.e. 0.5, 1, and 2 mg/ml from stock (20 mg/ml in PBS containing 40 mg/ml Pluronic F-68 as stabilizer) were prepared in pre-warmed (37 °C) growth media. Similarly, only Pluronic F-68 was diluted in the growth media, to achieve the equivalent concentrations used for preparing nanoemulsions. The media of cells in the 96-well plate was replaced after 24 hours with nanoemulsion solutions and Pluronic F-68 solutions. After 48 hours and 72 hours incubation time at 37 °C, 5% CO_2, 10 µl of WST-1 cell proliferation reagent was added, and the plate was incubated for an additional 2 hours. The absorbance of samples was then read according to the manufacturer protocol (420–480 nm). The percentage cell proliferation was reported relative to untreated cells (100% viability). Similar procedure was followed in order to determine the toxicity of curcumin and curcumin loaded nanoemulsions. Curcumin stock solution was prepared in DMSO, to achieve similar concentrations of curcumin present in the nanoemulsion samples. Two different concentrations of curcumin (i.e. 2 and 4 µg per 100 µl) in cell media was prepared and incubated for 48 hours and 72 hours to assess the cytotoxicity.

4 Results and Discussion

4.1 Synthesis of polymer

The polymer was synthesized via a well-known ROP route in bulk following the reported procedure (Figure 4). The synthesis methodology is straight-forward without a need of any special reaction setup and, therefore, this synthesis approach could be considered as industrial-friendly. The percentage conversion of monomer to polymer was calculated before purification by ^{1}HNMR by integrating the peak at 4.2 (monomer peak) and 4.8 ppm (polymer peak), which was found to be 90%. The unconverted monomer and catalyst were then washed out by methanol to obtain a pure polymer. Proton and carbon NMR of the polymer confirmed the synthesis and recovery of the pure polymer and the observed peaks matched with the reported values (Bansal, et al., 2015) (Figure 5). The higher value of calculated MW of the polymer (by ^{1}HNMR) to theoretical MW can be attributed to the presence of homopolymer chain (initiated by alcohol other than propargyl alcohol) in the sample. The similar phenomena were reported earlier by Bansal et.al., where the presence of a ring opened monomer δ-decalactone was responsible for producing an undesired homopolymer. Since, the MW by NMR was calculated by the number of protons at 4.8 ppm with respect to the peak of initiator at 4.6 ppm (Figure 5) and thus such misinterpretation of MW is highly possible. For this reason, Mn by SEC was found to be almost half of the MW by NMR with low PD (Figure 6) and the similar result was also reported previously (Bansal, et al., 2015). Therefore, to avoid this discrepancy, the viscosity of the polymer as an additional parameter was also determined to control batch to batch variability in polymer synthesis. The shear rate vs shear stress curve suggested that the PDL polymer is a Newtonian fluid and the infinite rare viscosity was found to be 62.20 Pa.s. Nevertheless, it was assumed that the presence of undesired homopolymer will not affect the nanoemulsion preparation but will certainly avoid an additional purification step.

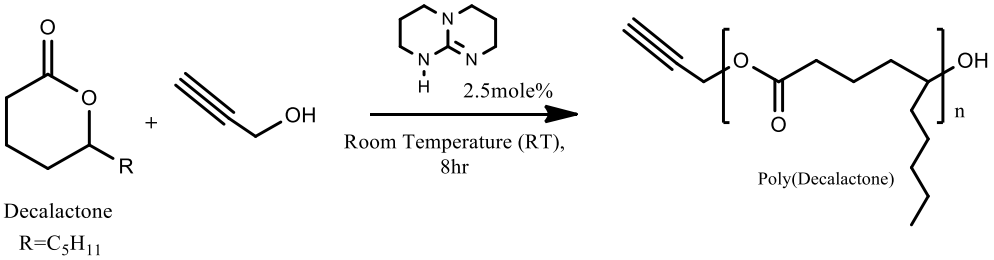

Figure 4. Synthesis scheme of poly(decalactone) polymer.

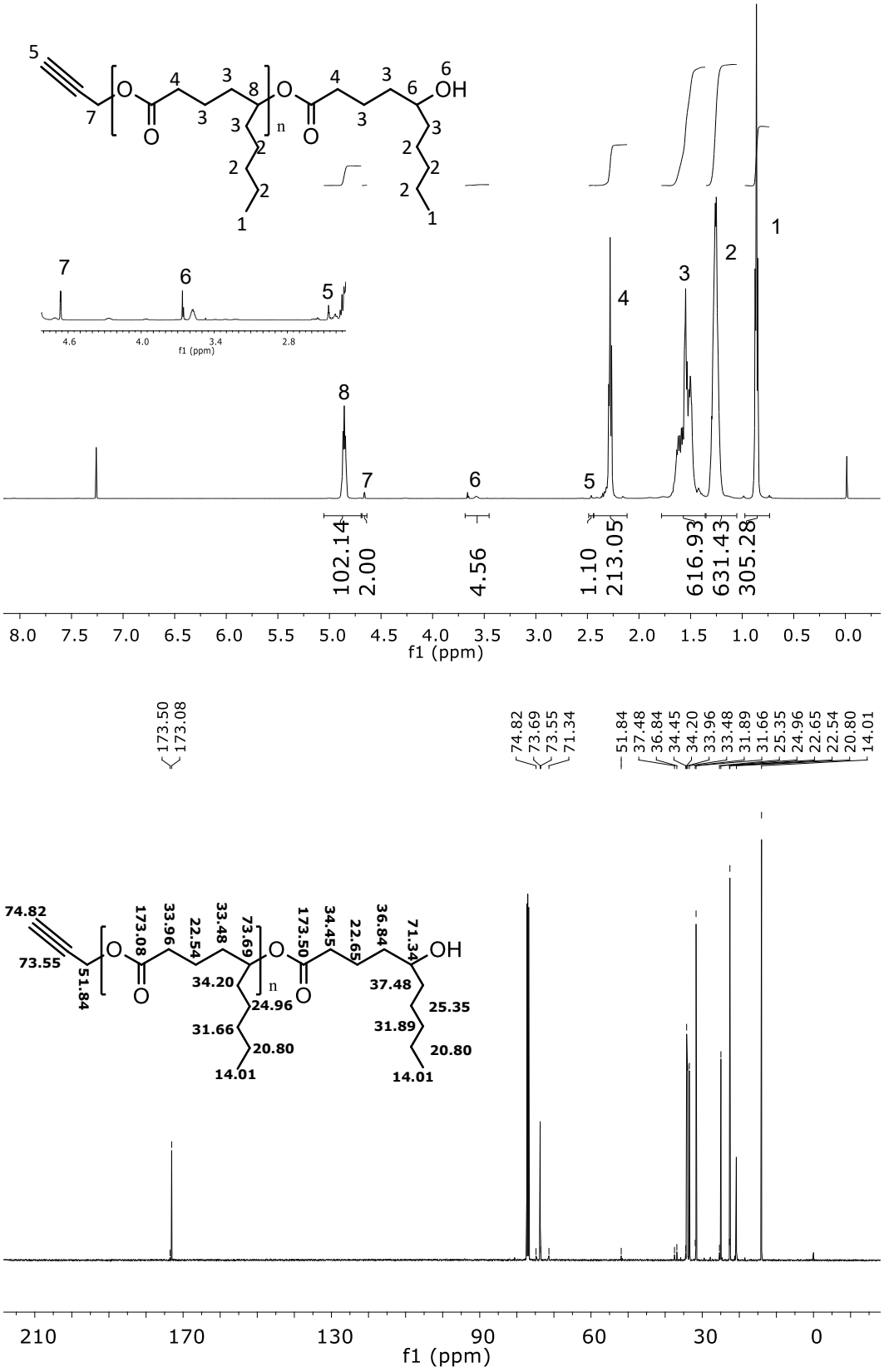

Figure 5. ^{1}HNMR and ^{13}CNMR (bottom) of poly(decalactone) in $CDCl_3$.

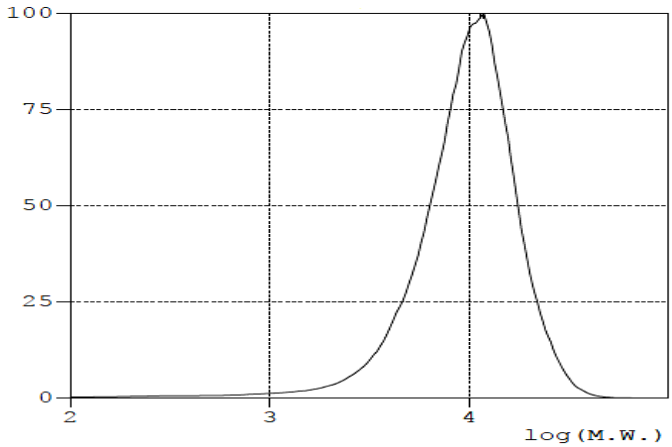

Figure 6. SEC trace of poly(decalactone) against polystyrene standard using THF as eluent.

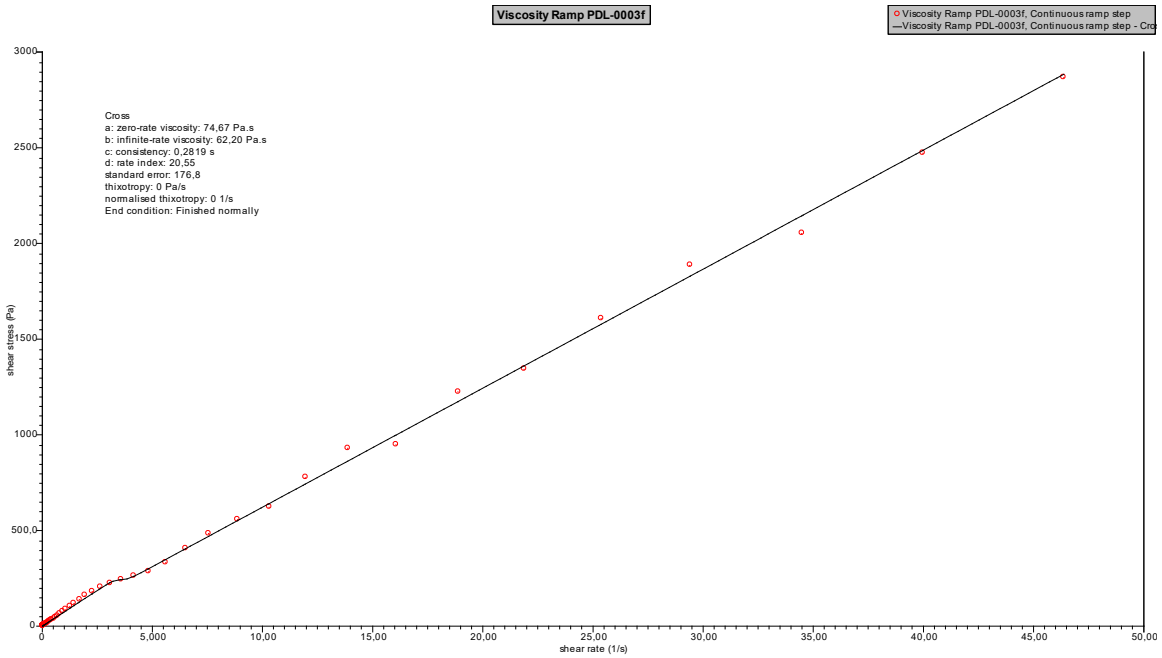

Figure 7. Shear rate versus shear stress curve of the PDL polymer.

4.2 Size of nanoemulsion droplets

The droplet size in the emulsion was analysed by dynamic light scattering (DLS) and TEM after appropriate dilutions. The Z-average sizes obtained by DLS were less than 200 nm, with low polydispersity index (PDI), except for the curcumin loaded sample, where the size observed was 200 nm (Table 1,Figure 8). It can be presumed from the results, that the size and polydispersity of Pluronic micelles was reduced, when the core was filled with the PDL polymer. The size reduction of Pluronic micelles after addition of a highly hydrophobic oily

polymer, suggests a strong interaction between PDL and PPO block, leading to reduction in overall micelle size and PDI. A similar observation has been made earlier, when hydrophobic compounds were loaded into Pluronic micelles (Grillo, Morfin, & Prévost, 2018; Sharma & Bhatia, 2004). However, as expected, an increment in size was observed after loading of hydrophobic drugs within the nanoemulsion. The size and surface morphology of blank, and curcumin loaded nanoemulsion was further examined using TEM. The images suggested that the emulsion droplets are spherical in shape, with a size less than 200 nm (Figure 10 and Figure 11).

Table 1. Z-average (d.nm) PDI & zeta potential (mV) with standard deviation (SD), ND - not determined

Sample	Z-Average Size (SD)	Polydispersity index (SD)	Zeta Potential (SD)
Blank	136.2 (2.060)	0.076 (0.017)	-2.05 (0.128)
Pluronic F-68	158.4 (1.587)	0.128 (0.021)	ND
Cyclosporin A	162.3 (2.170)	0.063 (0.020)	4.84 (0.418)
Prednisolone	170.6 (0.379)	0.073 (0.020)	-2.02 (1.01)
Curcumin	268.0 (13.99)	0.078 (0.059)	-11.3 (0.666)

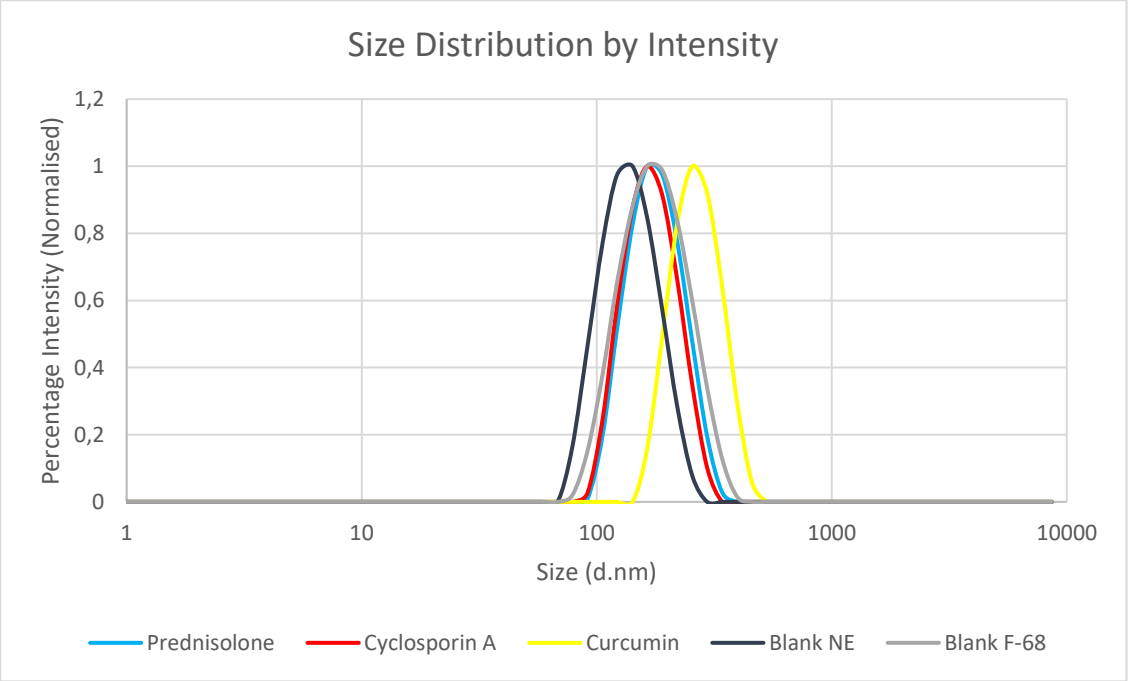

Figure 8. Size distribution by intensity for nanoemulsions with prednisolone, cyclosporin A, curcumin, blank nanoemulsion (NE) and blank Pluronic F-68 (F-68), displayed with normalised values.

The zeta potential distribution of blank and drug loaded nanoemulsions was measured in order to predict the stability, and to see the effect after drug loading (Figure 9). As shown in Table 1, no change in zeta potential was observed after prednisolone loading, however the charge shifted to positive with cyclosporin A and to negative after curcumin loading. This shift can be attributed to the structure of drugs, where the presence of amine groups (cyclosporin A) and hydroxyl groups (curcumin) influence the overall surface charge. Generally, zeta potential values, which exceed 30 mV (positive or negative) is ideal for a stable colloidal system. However, the zeta value close to zero in this study indicates that the nanoemulsion is sterically stable, rather than electrostatically stable, due to the presence of PEO blocks of Pluronic on the surface of the oil droplets.

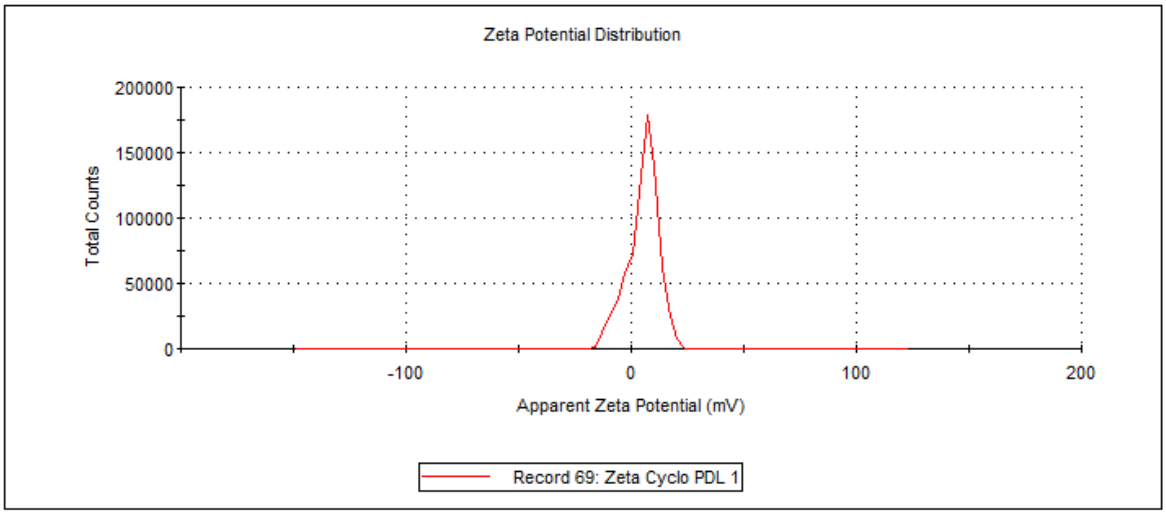

Figure 9. Zeta potential (mV) for a nanoemulsion with cyclosporin A.

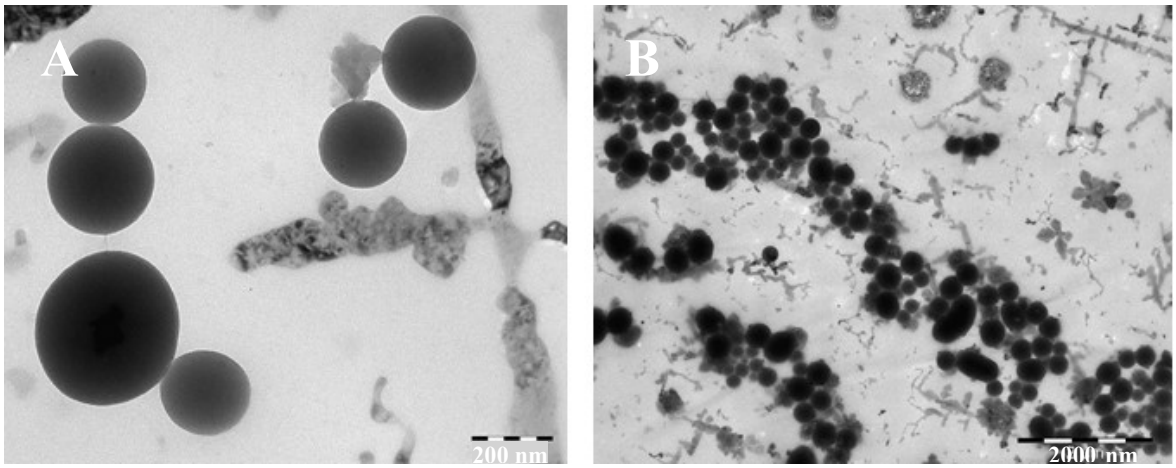

Figure 10. TEM images of non-loaded nanoparticles, close-up with scale of 200 nm (A) and (B) with scale of 2000 nm.

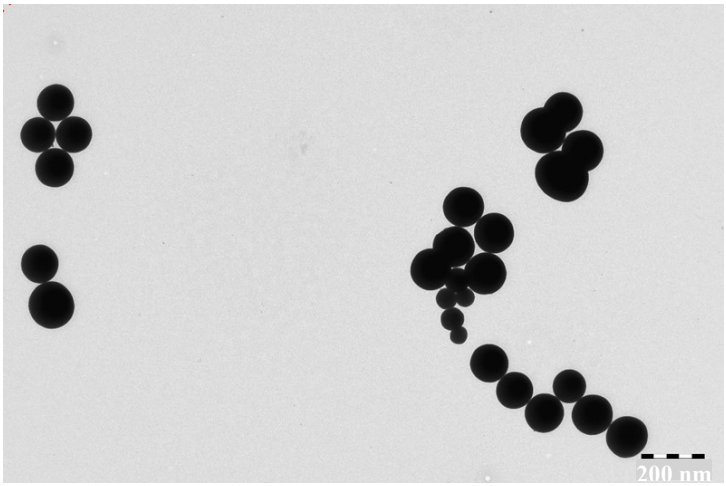

Figure 11. TEM image of a curcumin loaded nanoemulsion, with scale of 200 nm.

4.3 Drug loading in nanoemulsions

After preparation of the three samples, (drug in water, with Pluronic F-68, and nanoemulsion) undissolved drugs were removed by filtration. After filtration, on visual inspection it was observed that the drug in water and Pluronic F-68 samples were clear, while the nanoemulsion samples were a bit hazy (Figure 12).

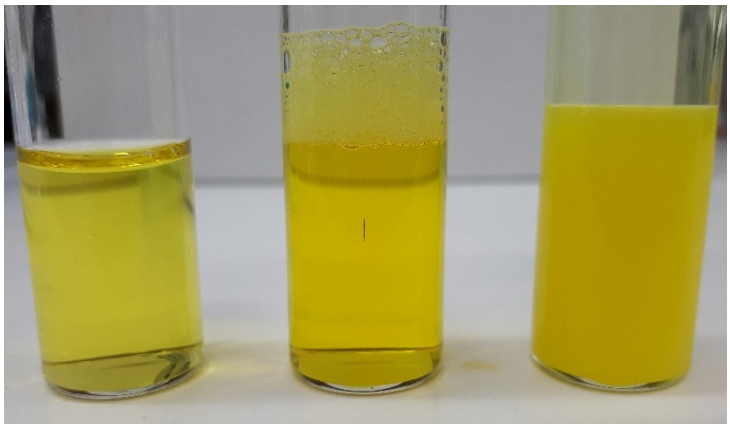

Figure 12. From left to right; curcumin in water, with Pluronic, and as a nanoemulsion.

In order to determine the drug content, nanoemulsion samples were analysed by UV-Vis, or by HPLC after appropriate dilutions. The amount of drug present in samples were calculated, using the calibration curves that had been prepared for this. Figure 13 shows the UV spectra collected for the calibration curves for the four drugs that were analysed by UV. Prednisolone was analysed from the peak at λmax 247 nm, carvedilol at λmax 242 nm, griseofulvin at λmax 295 nm, and curcumin at λmax 423 nm. From Figure 14 the trend lines and the regression functions used for the drug amount calculations can be observed.

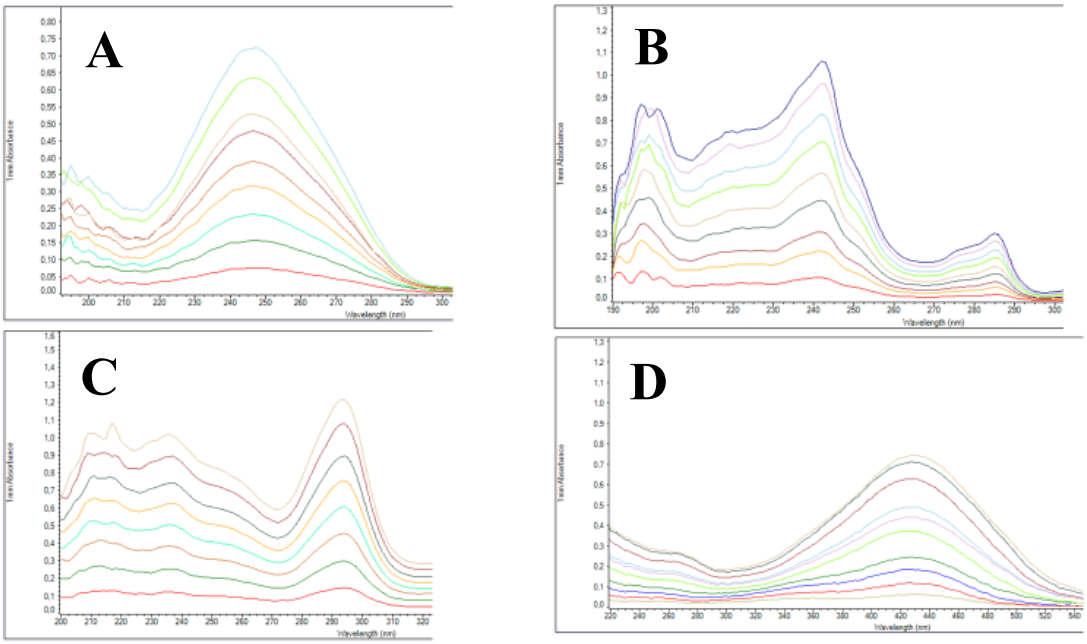

Figure 13. UV-Vis spectra of prednisolone (A), carvedilol (B), griseofulvin (C), and curcumin (D).

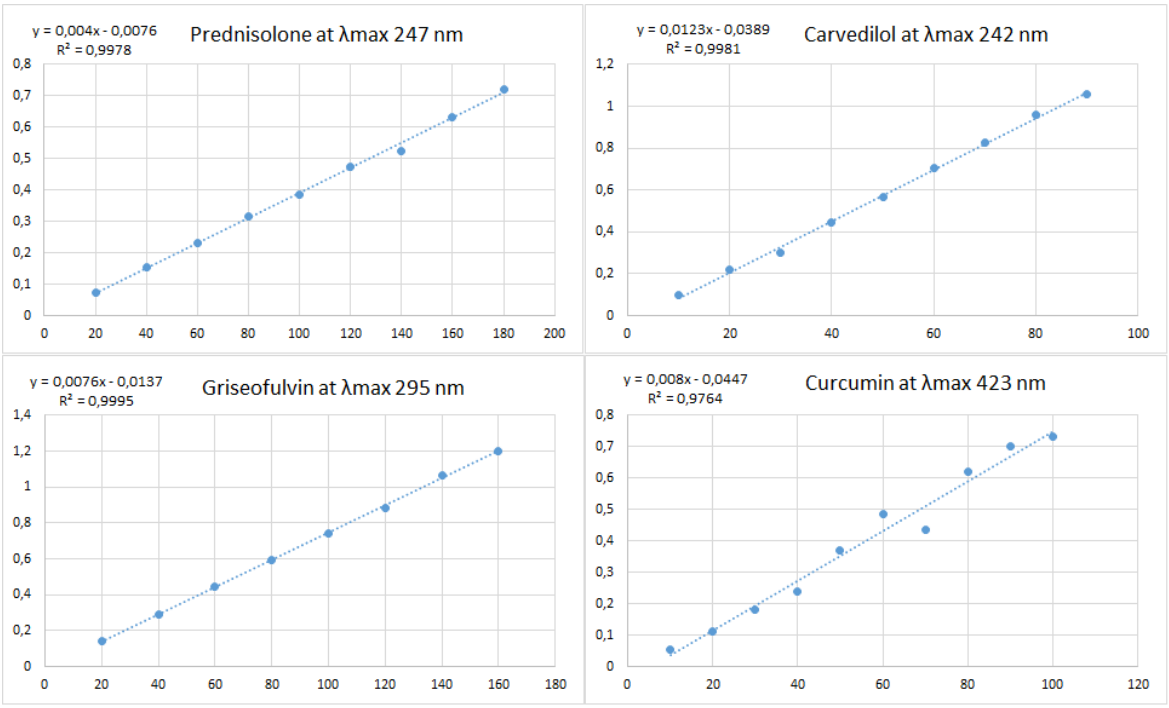

Figure 14. Calibration curves of prednisolone, carvedilol, griseofulvin, and curcumin.

Figure 15 shows the curve of one of the HPLC runs for the calibration curve and the plotted calibration curve. The peak that occurs at around 2.83 minutes was the one used for making a calibration curve and calculating the amount of cyclosporin A in the samples.

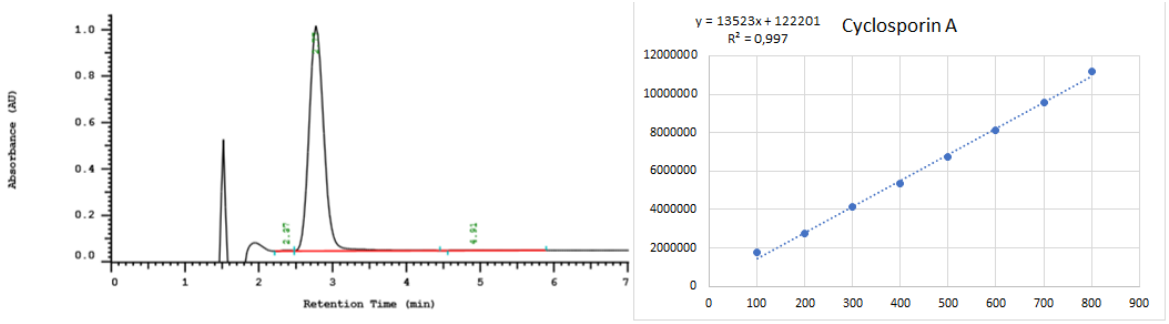

Figure 15. HPLC of cyclosporin A 500 µg/ml and the calibration curve made from the obtained results.

For comparison, the amount of drug in Pluronic micelles samples were also calculated, and the results obtained are shown in Figure 16. As expected, the nanoemulsions were capable of enhancing the aqueous solubility of all tested drugs as well as demonstrating superior performance compared to Pluronic F-68 micelles. The results suggested that the aqueous solubility of prednisolone, curcumin, carvedilol, cyclosporin A, and griseofulvin has been increased by 3, 5, 6, 9, and 10 times respectively. The nanoemulsions managed to increase the solubility of the drugs by three up to ten times compared to Pluronic micelles.

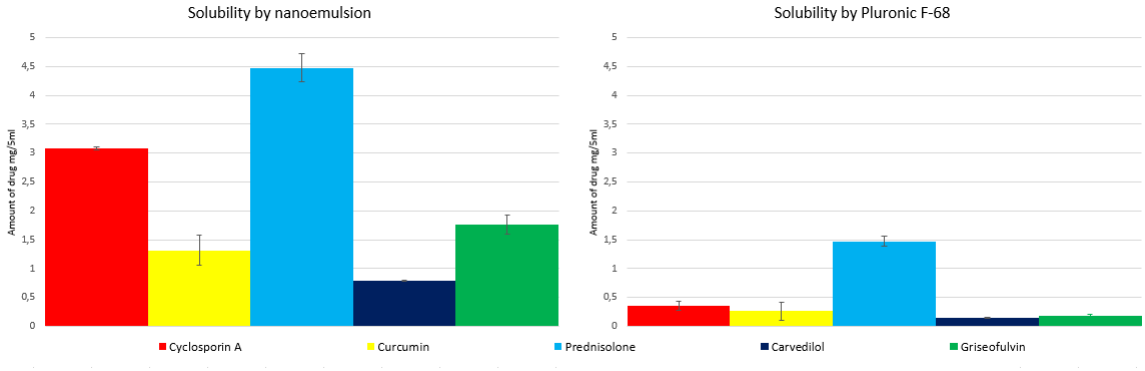

Figure 16. Comparison between the solubility of the drugs by nanoemulsion and by Pluronic F-68.

In order to calculate the percentage of drug content in the samples, the amount of drug loaded into the nanoemulsion was divided by the amount of polymer used, as shown in the formula in the methods section. Prednisolone, being the most water-soluble drug, was the easiest to load into the polymer, with a drug content at 17.9%, close to the maximum 20%. The nanoemulsions with the drugs cyclosporin A, curcumin, carvedilol, and griseofulvin had drug content percentages of 12.3, 5.3, 3.1, and 7.0 respectively. Naturally, the corresponding results for Pluronic micelles are also here three to ten times lower, which can be observed in Figure 17. In Figure 18 the calculated encapsulation efficiency can be observed. This graph shows how many percent of the drug, used to prepare the mixture, is actually loaded.

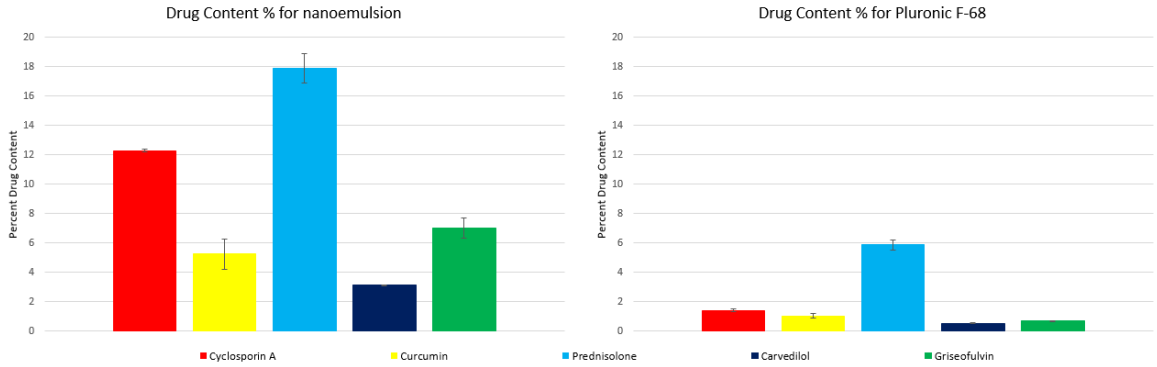

Figure 17. Percent drug content for each sample.

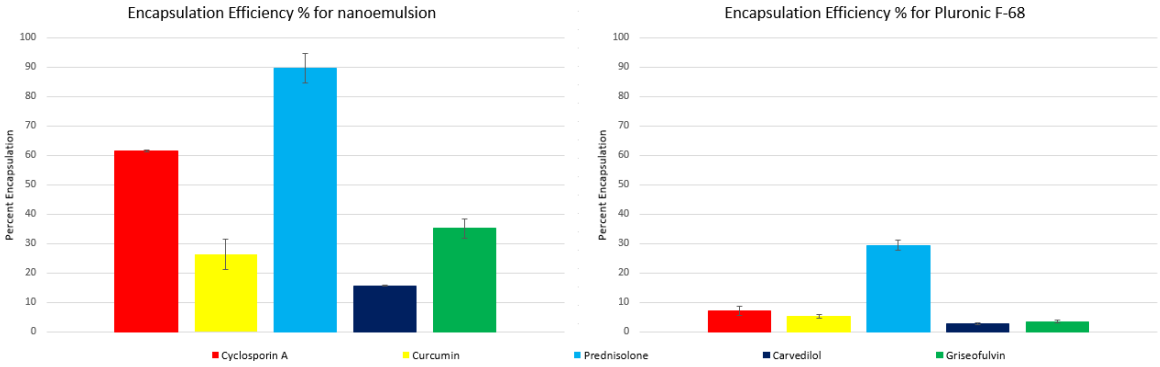

Figure 18. Encapsulation efficiency for the drugs.

An earlier study that investigated how the solubility of carvedilol could be improved by making nanoemulsions was carried out by Drais and Hussein (2015). Their most promising nanoemulsion formulation consisted of 10% peppermint oil, 20% Tween 80, 10% ethanol, and 60% distilled water. They found a significant increase in solubility of the carvedilol in nanoemulsion, compared to the pure drug. The best nanoemulsion had an estimated drug content of 99%. With griseofulvin, non-aqueous nanoemulsions have been prepared earlier, and the research showed that the formulation improved the stability of griseofulvin (Jadhav, Kate, & Payghan, 2015). However, the research did not present any drug content results in comparison to a non-nanoemulsion. Regarding prednisolone no previous studies with nanoemulsions were found.

Cyclosporin A has undergone several studies involving nanoemulsions previously. One study being a preparation of a pellet-based, solid self-nanoemulsifying drug delivery system (SNEDDS). A fluid-bed coating technique led to a faster redispersion rate of the liquid SNEDDS, and the loading of cyclosporin A did not affect the redispersion rate (Lei, et al.,

2011). Another study, also investigating cyclosporin A SNEDDS found out that it can affect the emulsification rates and the physical properties in a positive way (Zidan, et al., 2007). Curcumin has also been involved in a number of studies about nanoformulations (Bansal, Gupta, Rosling, & Rosenholm, 2018). One study on nanoemulsions managed to obtain an encapsulation efficiency of curcumin at 90%, suggesting that nanoencapsulation of highly lipophilic and unstable compounds can be advantageous (Sari, et al., 2015).

4.4 Stability

The stability of prepared nanoemulsions was observed by applying force (centrifugation) in order to accelerate emulsion breakage. No sign of phase separation was observed after centrifuging the samples for 30 minutes at 10,000 rpm (Figure 19). In addition, long-term storage stability was checked, by storing two drug-loaded nanoemulsion samples for three months at room temperature and at 50 °C. Samples were withdrawn every 30 days and analysed to observe the change in size, charge, and drug content. No significant difference was observed in any of the parameters.

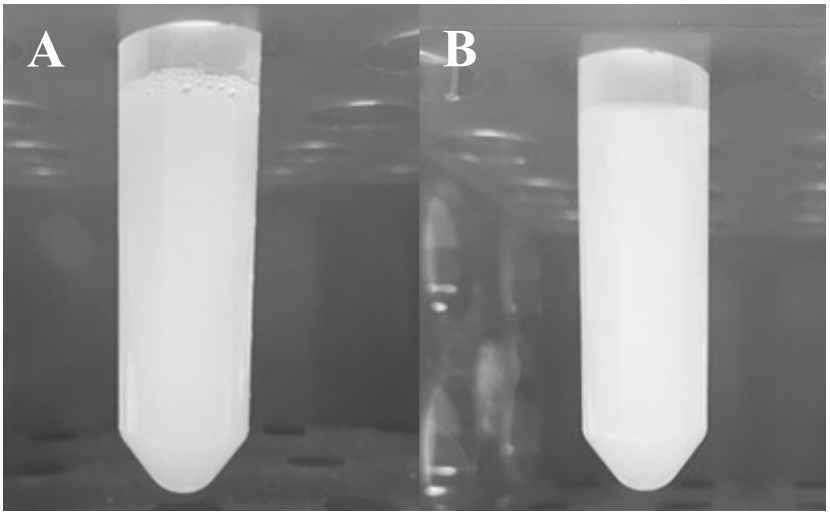

Figure 19. Nanoemulsion before (A) an after (B) centrifugation.

Figure 20 presents the UV absorption of samples stored at different conditions from day one to day 90. No significant change was observed, suggesting that the nanoemulsion is capable of protecting the loaded drug from degradation.

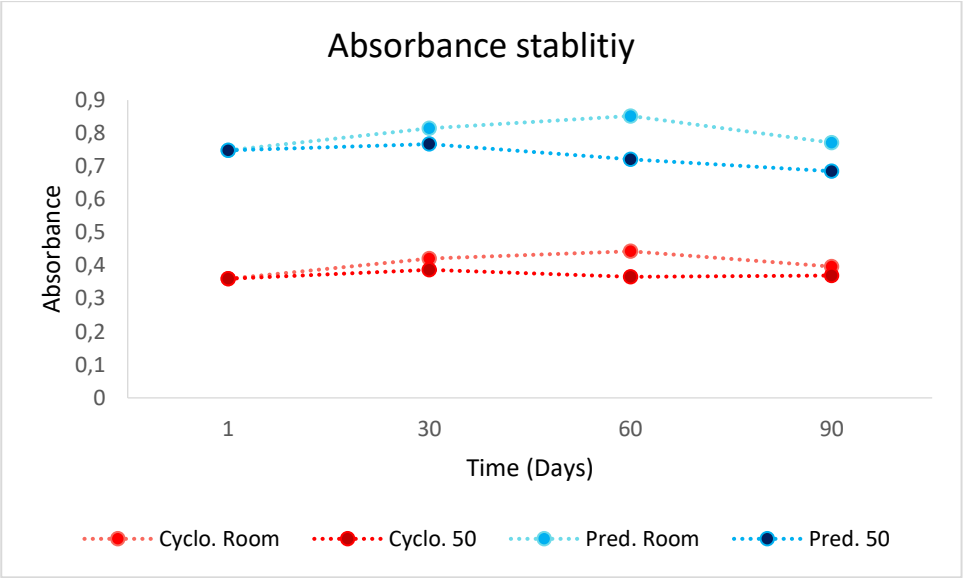

Figure 20. Stability from variations in collected UV-Vis spectra for prednisolone (Pred.) and cyclosporin A (Cyclo.) stored at 50 °C (50) and at room temperature (Room).

The zeta potential value varies over the three months. This can be seen from Figure 21. Since the zeta potential values could never be considered as stable, it is difficult to conclude anything from the results. One reason for the variation in zeta potential could be that the analysis was carried out using Milli-Q water, not knowing the pH values for the samples at the different measuring times.

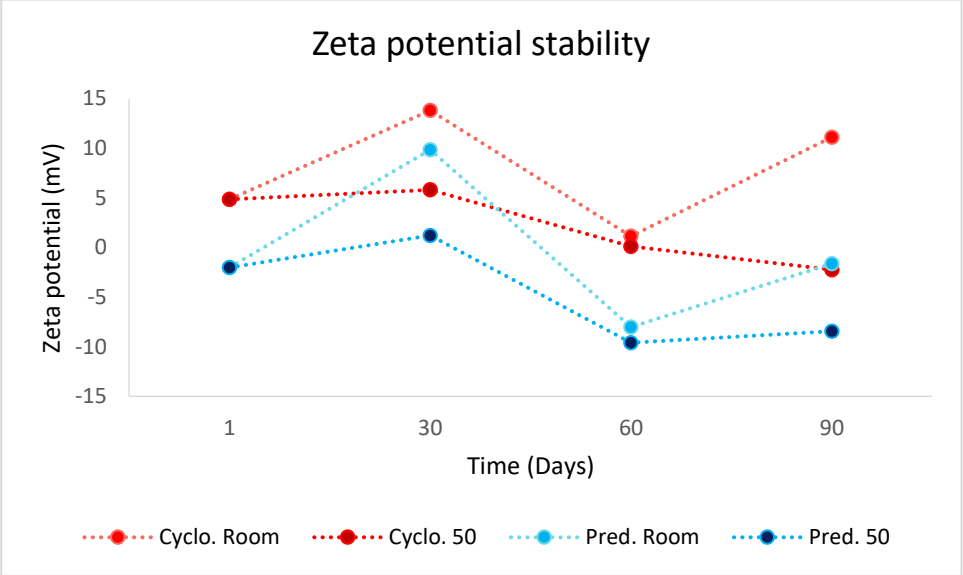

Figure 21. Stability expressed as variations in zeta potential for cyclosporin A (Cyclo.) and prednisolone (Pred.) for samples that has been stored in room temperature (Room) and at 50 °C (50).

From Figure 22 it can be observed how the size of the nanoparticles changes over the three months. The significant change in size can be observed after three months of storage at room temperature. Although, the maximum size recorded is less than 220 nm, with PDI less than 0.15. The results clearly suggest that the nanoemulsions are stable up to at least three months regarding change in size (coalescence, aggregation). When inspecting the samples visually, after 3 months of storage, there was no sign of phase separation, or gravitational separation in any of the samples. Surprisingly, the samples show greater uniformity in size at 50°C, compared to the samples stored at room temperature. This could be due to the melting point of Pluronic F-68 (50–55°C), which makes the polymer more flexible at higher temperatures, and thus avoiding further aggregation and sedimentation.

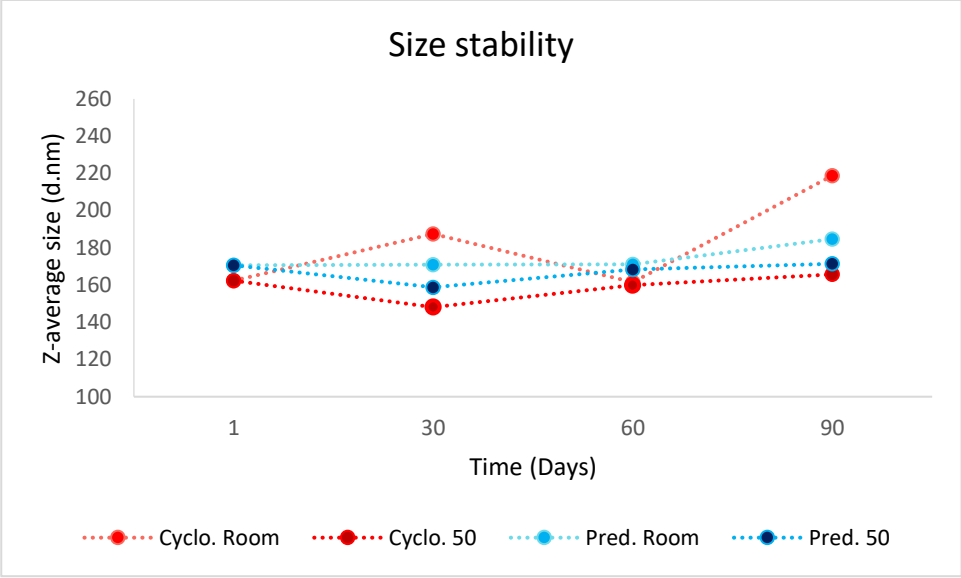

Figure 22. Change in size over three months for cyclosporin A (Cyclo.) and prednisolone (Pred.) stored in room temperature (Room) and at 50 °C (50).

4.5 Haemolysis

The haemolysis is usually dose-dependent, as increasing concentrations of the test materials correspond with higher levels of haemolysis (Evans, et al., 2013). Correspondingly, in this study, the percentage of haemolysis increased with the increasing concentrations of nanoemulsion. After one hour of incubation, approximately 10% of haemolysis was

observed in the samples containing 40 mg/ml nanoemulsion with RBCs, whereas haemolysis of less than 5% was observed in the samples with lower concentrations. In contrast, after 24 hours of incubation, rupture of RBCs was above 10% even for concentrations as low as 10 mg/ml (Figure 23 & Figure 24). From the results, it can be concluded that the nanoemulsion containing 1 mg/ml of the PDL polymer is non-haemolytic and can be administered through IV route without eliciting any side effects.

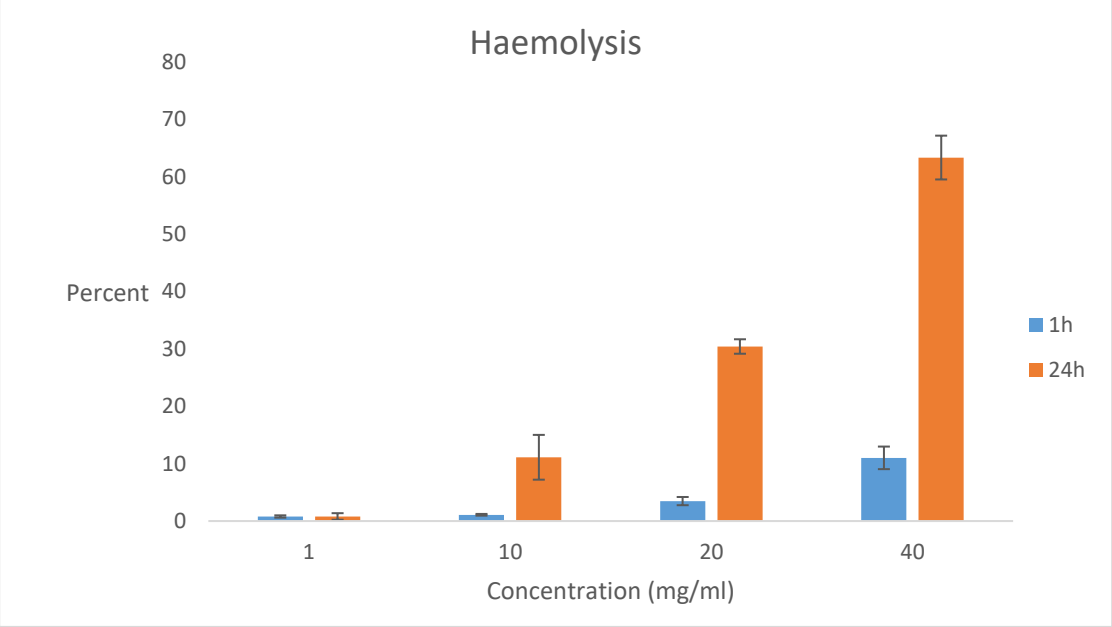

Figure 23. Percent of haemolysis at all concentrations after 1 and 24 hours.

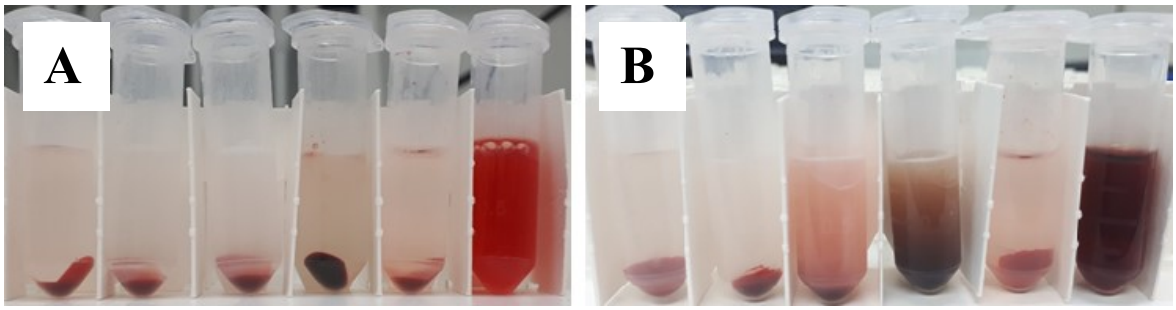

Figure 24. Haemolysis samples after (A) 1 h and (B) 24 h, after centrifugation. From left to right 1, 10, 20, and 40 mg/ml, negative and positive control.

4.6 Cell study

The percentages of cell proliferation after treatment, with different concentrations of nanoemulsion and Pluronic F-68, is shown in Figure 25. From the results, it can be concluded that not more than 10% of cell death was triggered by nanoemulsion up to concentrations of 1 mg/ml after 48 hours and up to concentrations of 0.5 mg/ml after 72 hours of incubation. Both samples demonstrate concentration and time-dependent toxicity. However, no significant difference in the toxicity profile was observed between the nanoemulsion and Pluronic F-68 micelles alone. It should be noted that the ratio of polymer to Pluronic used for preparing nanoemulsions for cytotoxicity studies is much smaller than the ratio used for preparing nanoemulsions for drug loading. The reason for using a reduced amount of Pluronic in the cell study is associated with the cytotoxicity of Pluronic itself. As demonstrated in Figure 25, almost 40% of cell death was observed with Pluronic F-68, at concentrations of 4 mg/ml (i.e. 400 µg in 100 µl of cell media), and 100% cell death was observed at 6 mg/ml (data not shown).

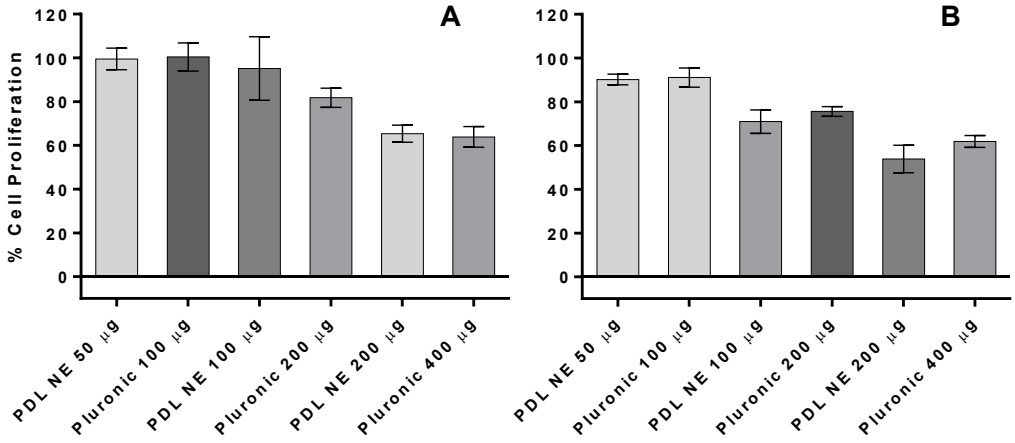

Figure 25. Percentage cell proliferation calculated by WST-1 assay after treatment with different samples at time point (A) 48 h and (B) 72 h. Data represents average of three measurements with standard deviation.

The reported cytotoxicity data for Pluronic F-68 did not demonstrate a toxicity profile above 1 mg/ml, and hence, these results offer a selection of right concentrations for future cell studies. A stable emulsion was obtained using this ratio, as determined by centrifugation test and size analysis. It has been proposed that the polymer globules could be completely coated by Pluronic, and cells might only be interacting with the hydrophilic part, because the

concentration of Pluronic used was above its critical micelle concentration (CMC) (~ 0.3 mg/ml). It can be concluded that PDL does not induce any cytotoxicity by itself in tested formulations. However, these results clearly suggest, that the cytotoxicity of the Pluronic F-68 surfactant reduced considerably, when the PDL polymer was loaded in the core. This could be related to the decrease in CMC of Pluronic F-68 in the presence of a hydrophobic polymer, which induce hydrophobic interaction (Rapoport, 1999; Shirahama & Kashiwabara, 1971). The toxicity of surfactants is usually associated with the amount of surfactant monomer presents below CMC, and thus, a decrease in CMC can be directly related to a decrease in toxicity (Partearroyo, Ostolaza, Goñi, & Barberá-Guillem, 1990).

The effect of toxicity of curcumin on MDAMB-231 cancer cells was evaluated after being encapsulated in the nanoemulsion. The curcumin cytotoxicity found on MDAMB cells is similar to previously reported results (Lv, et al., 2014). The toxicity results were compared to toxicity results for pristine curcumin, suggesting that the loaded curcumin demonstrate a slightly higher toxicity, notable after 72 hours of incubation (Figure 26). The superior toxicity of curcumin encapsulated in the nanoemulsion can be attributed to the enhanced stability of loaded curcumin, compared to free drug (Bansal, Gupta, Rosling, & Rosenholm, 2018).

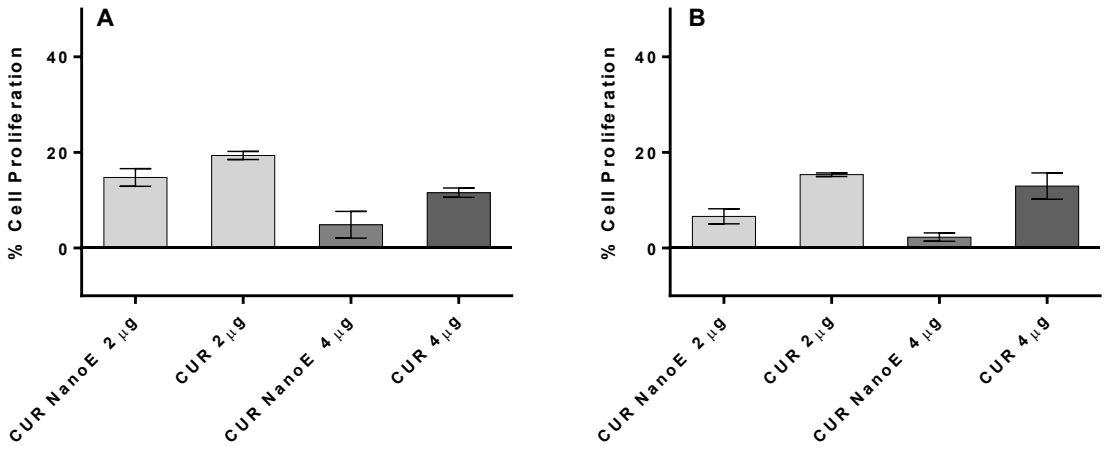

Figure 26. Percentage of cell proliferation (MDAMB-231) calculated by WST-1 assay, after treatment with different curcumin samples at time point (A) 48 h and (B) 72 h. The data represents the average of three measurements and the standard deviation. The concentrations given are for 100 µl of cell media (CUR NanoE- curcumin loaded nanoemulsion, CUR- pristine curcumin).

5 Conclusion

In this study, a nanoemulsion was successfully fabricated using hydrophobic oily polymer PDL, instead of traditional oil, and Pluronic F-68, as surfactant. The droplet size was found to be less than 200 nm, except in the curcumin sample where possible coalescence is expected based on the surface charge. Drug loading study suggests that the nanoemulsion samples were able to increase the aqueous solubility of all tested drugs 3–10 times compared to the Pluronic micelles samples. Polymeric nanoemulsions showed good stability against force separation (centrifugation), and good stability in long-term storage at room temperature and 50°C in terms of size, breaking, and drug degradation. The nanoemulsion demonstrated a time and concentration-dependent cell toxicity; however, there was no significant difference to the Pluronic micelles alone. It could be concluded that the PDL polymer itself did not induce any cytotoxicity. The haemolysis study also showed a higher percentage of haemolysis of the red blood cells at the higher concentrations of the nanoemulsion. It would be interesting to further study the toxicity of PDL with a lower amount of Pluronic F-68 or with a completely different surfactant.

The use of the PDL polymer in making nanoemulsions is a new approach, and the results obtained cannot be directly compared to previous studies. Nevertheless, using PDL instead of a natural oil as the oil phase in nanoemulsions is a promising approach. However, this approach can be seen as enhancing the stability and drug loading capacity of Pluronic micelles, as reported previously (Rapoport, 1999).

Further studies are needed with the polymer to confirm its suitability for nanoemulsions, to investigate whether it would improve delivery of poorly soluble drugs, and whether it could be developed into practical drug delivery systems.

6 Summary in Swedish – Svensk sammanfattning

Nanoemulsioner av förnybara polymerer för att förbättra levereringen av läkemedel

Låg biotillgänglighet är ett allmänt problem bland nya läkemedelsmolekyler idag. Det kan innebära att molekylerna har låg löslighet eller låg permeabilitet, eller både och. Det finns flera tillvägagångssätt för att försöka förbättra lösligheten för läkemedelsmolekylerna, som att modifiera molekylerna fysiskt eller kemiskt, och i denna studie har det gjorts med nanoemulsioner. Nanomedicin är ett område där läkemedel med hjälp av nanopartiklar försöker förbättra lösligheten, farmakokinetiken eller målstyrningen hos läkemedel, minska toxiciteten, öka säkerheten eller på andra sätt förbättra effektiviteten av medicinerna. Nanopartiklar inom läkemedelsadministrationssystem kan finnas i form av bland annat miceller, liposomer, inorganiska nanopartiklar och polymeriska nanopartiklar. Idag utvecklas även komplicerade nanopartikelsystem, som till exempel ska kunna leverera läkemedel direkt till cancertumörer.

Nanoemulsioner består av små partiklar av en oblandbar vätska dispergerad i en annan oblandbar vätska, vanligen en oljefas och en vattenfas. Ytterligare tillsätts ofta en eller flera ytaktiva ämnen, även kallade surfaktanter. Surfaktanternas roll är att stabilisera emulsionen genom att hålla faserna i en suspension med små partiklar och undvika agglomerering. Nanoemulsioner går att formulera till flertalet beredningsformer, så som tabletter, sprayer, injektionsvätskor, salvor och krämer. Att partiklarna i nanoemulsionerna är av så liten storlek gör att den relativa ytarean blir mycket hög, detta kan ge fördelar som långsiktig fysiskstabilitet och en ökad biotillgänglighet. De fem läkemedelssubstanserna som användes i denna studie för att undersöka nanoemulsionens förmåga att öka lösligheten var; cyklosporin A, griseofulvin, karvedilol, kurkumin och prednisolon. Syftet med detta projekt var att utveckla och karakterisera ett stabilt polymernanoemulsionssystem via enkel metod för läkemedelsleveransapplikationer.

Nanoemulsionerna framställdes genom att blanda läkemedel med lösningsmedel och poly(δ-decalactone) (PDL) polymer för att erhålla oljefasen, medan vattenfasen bestod av Milli Q vatten och surfaktanten Pluronic F-68. Det finns flera olika metoder för att framställa nanoemulsioner, och en del av dem kan vara väldigt kostsamma. Tiden en nanoemulsion hålls stabil kan i en del fall vara rätt så kort, eftersom olika faktorer som till exempel

gravitationskraften gör att emulsionen separerar. En fördel med att använda en syntetiskt framställd polymer som oljefas är att den inte härsknar som till exempel vegetabiliska oljor gör. Polymeren syntetiserades från en monomer δ-decalactone via en ringöppningspolymerisation. I denna studie var framställningsmetoden en simpel metod där oljefasen, med lösningsmedel och läkemedel adderades en droppe i taget till en vattenfas som även innehöll surfaktant under magnetomröring. Som referens framställdes även prover utan polymeren och prover utan polymer och Pluronic F-68. Proverna fick sedan stå under omrörning i minst tre timmar, och lämnades sedan med korken av under natten för att lösningsmedlet skulle avdunsta. De färdiga proverna filtrerades, så att läkemedel som ej lösts upp avlägsnades. Analyser för absorbans, celltoxicitet, hemolys, storlek och stabilitet gjordes för nanoemulsionerna.

Nanoemulsionernas koncentration av läkemedel uppskattades genom att analysera utspädda prover med hjälp av ultraviolettspektroskopi eller högupplösande vätskekromatografi (HPLC), och beräkna koncentrationerna utgående från framställda kalibreringskurvor. Nanoemulsionerna innehöll en högre koncentration läkemedel än de jämförande prover som inte innehöll polymeren, ökningen var mellan tre och tio gånger för de fem läkemedel som användes. Genom analys med dynamisk ljusspridning (DLS) och transmissionselektronmikroskopi (TEM) kunde det bekräftas att partiklarna i emulsionerna var av nanostorlek. Storleksfördelningen visade att majoriteten av nanopartiklarna var av en storlek kring 200 nm och de största enskilda partiklarna var kring 500 nm. Hemolys- och celltoxicitetsstudierna gjordes för att undersöka om nanoemulsionerna skulle vara skadliga för kroppen. I hemolysstudien fanns det ett samband där proverna med högre koncentrationer av nanoemulsioner orsakade en högre andel hemolys, vilket innebär att en större andel av de röda blodkropparna som analyserades brast. Även i celltoxicitetsstudien orsakade de högre koncentrationerna av nanoemulsionerna och Pluronic en högre procentuell celldöd. I både hemolys- och celltoxicitetsstudierna kunde det även observeras att en längre förvaring av proverna orsakade en högre procentuell hemolys respektive celldöd.

Stabilitetsstudier gjordes genom att centrifugera en nanoemulsion i 30 minuter, vilket inte orsakade någon separering. Stabiliteten undersöktes även genom att förvara två läkemedelsladdade nanoemulsioner i tre månader i rumstemperatur (20 ± 2 °C) och

inkuberade vid 50 ± 2 °C. Under de tre månaderna kunde inga trender utifrån resultaten i vare sig storleksfördelning eller laddning observeras, ingen tydlig separering i nanoemulsionerna hade heller uppstått under denna tid. Även om nanoemulsionerna var stabila erhölls låga värden när deras zeta-potential undersöktes (under 15 mV), stabila zeta-potentialvärden för kolloidala system brukar anses vara över 30 mV, antingen positivt eller negativt. Detta tyder på att emulsionerna är stabila genom sterisk stabilisering snarare än genom elektrostatisk repulsion.

Att använda PDL polymeren för att framställa nanoemulsioner är ett nytt tillvägagångssätt och resultaten som erhållits kan inte direkt jämföras med tidigare studier. Vidare studier borde utföras för att undersöka hur polymeren passar till nanoemulsioner, huruvida den förbättrar leveransen av läkemedel och kan fungera som läkemedelsbärare.

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