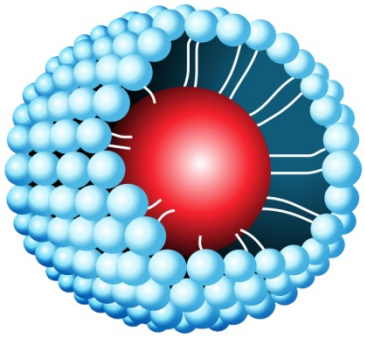
A thesis for the degree of Master of Science in Pharmacy

Novel Block Copolymer Self-Assembled Micelles as Nanocarriers for Drug Delivery:
Preparation, Characterization and *In Vitro* Evaluation



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ABSTRACT

Novel block copolymer self-assembled micelles as nanocarriers for drug delivery: preparation, characterization and *in vitro* evaluation.

The continuous search for new drug delivery systems is driven by the ardour to maximize therapeutic activity while minimizing negative side effects. Particularly, much attention is now being paid to amphiphilic polymeric micelles as nanocarriers for drug delivery systems. Block copolymer micelles as drug carriers are able to provide highly desirable advantages including increased solubilization of poorly soluble drugs. Poor aqueous solubility is a big obstacle in drug development. It poses such a serious problem that some pharmaceutical companies decide to exclude poorly soluble compounds very early in their screening process regardless of how active these compounds are.

It has repeatedly been shown that polymeric micelles can increase the solubility of poorly aqueous soluble drugs. Compared to other drug carriers, micelles can be obtained in an easy and reproducible manner on a large scale. The nano-size of the polymeric micelles not only makes them ideal drug delivery carriers as they escape from renal exclusion but also enhances their vascular permeability.

The aim of this research project was to explore the solubilizing potential of a novel block copolymer, Boc-(Leu-Leu-Ala-Lac)$_5$-Leu-Phe-O-PEG$_{4000}$-OH, also named DP7, through micellization of poorly soluble drugs in order to improve their bioavailability. Another objective was to characterize the micelles and to evaluate if fatty acids, such as stearic acid, could improve the efficacy of DP7 micelles.
The results show that DP7 self-aggregate and form nano-size micelles which greatly enhance the solubility of fenofibrate, which is a very poorly soluble drug in aqueous solutions. It was also observed that stearic acid enhanced the drug entrapment efficiency of the micelles but the inclusion of stearic acid did not result in slow drug diffusion from the micelles in *in vitro* studies.

Future studies will determine the behaviour of DP7 micelles *in vivo* and their feasibility in drug targeting.

ÁGRIP

Mísellur sem myndast af sjálfsdáðum úr nýstárlegum bjálka-samfjölliðum sem nanó-flutningskerfi fyrir lyf: undirbúningur, eiginleikar og *in vitro* prófanir.

Stöðug leit er að nýjum burðarkerfum fyrir lyf með það fyrir augum að hámarka læknisfræðileg áhrif þeirra og minnka aukaverkanir. Síðustu misseri hafa tvíleysnar fjölliður (*e*. amphiphilic polymers) af nanóstærð verið rannsakaðar ítarlega sem burðarkerfi. Mísellur úr bjálka-samfjölliðum (*e.* block copolymers) hafa marga kosti sem burðarkerfi fyrir lyfjaefni. Mikilvægur eiginleiki þeirra er að auka leysni lyfjaefna í vatnslausnum en takmörkuð leysni í vatni er gríðarstórt vandamál í lyfjaþróun. Vandamálið er svo stórt, að mörg lyfjafyrirtæki hafna mjög torleystum efnasamböndum snemma í skimunarferlum þeirra óháð því hversu lyfvirk efnin kunna að vera.

Sýnt hefur verið fram á að mísellur úr fjölliðum geta aukið vatnsleysni torleystra lyfjaefna. Aðferðir við framleiðslu mísellna eru auðveldar og endurtakanlegar á stórum skala samanborið við önnur burðarkerfi. Vegna nanóstærðar þeirra komast þær hjá því að vera skildar út um nýru ásamt því að auka gegndræpi háræða.
Markmið verkefnisins var að rannsaka áhrif nýrrar bjálka-samfjölliðu, Boc-(Leu-Leu-Ala-Lac)$_5$-Leu-Phe-O-PEG$_{4000}$-OH sem einnig er kölluð DP7, á leysanleika torleystra lyfjaefna með myndun mísellna með það fyrir augum að auka aðgengi efnanna. Önnur markmið voru að rannsaka eiginleika mísellnanna sem og að athuga hvort fitusýrur, eins og sterínsýra, gætu bætt eiginleika þeirra.

Niðurstöður rannsókna sýna að DP7 þyrpast saman af sjálfsdáðum (*e*. self-aggregate) og mynda nanómísellur. Nanómísellurnar juku stórlega leysni fenófíbrats í vatnslausnum en fenófíbrat er mjög torleysanlegt í vatni. Sterínsýra jók getu mísellnanna til að innlima fenófíbrat en hún leiddi ekki til hægrar losunar þess úr kerfunum skv. *in vitro* rannsóknum.

Með frekari rannsóknum verður hægt að skýra virkni mísellna úr DP7 *in vivo* og ákvarða hvort þær geti komið að notum við markvissa lyfjagjöf til ákveðinna staða í líkamanum (*e.* drug targeting).

ABBREVIATIONS

AB	Assay buffer
ACSR	Acyl-CoA Synthetase Reagent
Ala	Alanine
BCS	Biopharmaceutics Classification System
Boc	Tert-butoxycarbonyl
CAC	Critical association constant
CMC	Critical micelle concentration
CsA	Ciclosporin A
CSM	Co-solvent method
CSO	Chitosan oligosaccharide
CSO-SA	Stearic acid grafted chitosan oligosaccharide micelles
DLS	Dynamic light scattering
DMSO	Dimethylsulfoxide
DP7	Boc-(Leu-Leu-Ala-Lac)$_5$-Leu-Phe-O-PEG$_{4000}$-OH
DSC	Differential scanning calorimetry
EE	Entrapment efficiency
EM	Enzyme Mix
FF	Fenofibrate
FR	Filtration rate
GRG	Guaranteed Reagent Grade
HPLC	High-performance liquid chromatography
JIS	Japanese Industrial Standard
Lac	Lactic acid
Leu	Leucine
MWCO	Molecular weight cut off
nm	nano meter
NM	Nanomizer
NME	New molecular entities
NMM	Nanomizer method
PA	Palmitic acid
PBS	Phosphate buffer solution
PCL-g-PVA	Poly(ε-caprolactone)-grafted-poly(vinyl alcol)

PdI	Polydispersity index
PEG	poly(ethylene glycol)
PEG-β-PCL	poly(ethylene glycol)-block-poly(ε-caprolactone)
PEO	poly(ethylene oxide)
Phe	Phenylalanine
PVP	poly(N-vinyl pyrrolidone)
RM	Reaction Mix
SA	Stearic acid
SD	Degree of amino substitution
T	Temperature
TFM	Thin film method
T_g	Glass transition temperature
T_m	Melting point

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TABLES

FIGURES

1. INTRODUCTION

The continuous search for new drug delivery systems is driven by the ardour to maximize therapeutic activity while minimizing negative side effects. Since Bader et al. proposed polymeric micelles as drug carriers in 1984 they have become one of the most promising modalities as drug carriers (Qiu, Wu & Jin, 2009). Particularly, much attention is now being paid to amphiphilic polymeric micelles as nanocarriers for drug delivering systems. Block copolymer micelles as drug carriers are able to provide highly desirable advantages. They are e.g. solubilization of poorly soluble drugs and thus increased bioavailability, reduced toxicity and other adverse effects, high loading capacity, nano-size, enhanced permeability across physiological barriers and they can also extend blood half life upon intravenous administration. Besides, micelles can be made marked for targeted drug delivery by attachment of a specific ligand on the outer surface. Being in a micellar core, the hydrophobic drug is well protected from possible inactivation caused by biological surroundings. Compared to other drug carriers, micelles can be obtained in an easy and reproducible manner on a large scale (W. Q. Chen et al., 2008; Torchilin, 2001).

Micelles belong to a large family of dispersed systems. Their viral-like size (< 100 nm (nanometer)) prevents their uptake by the reticuloendothelial system and facilitates their extravasations at leaky sites of capillaries, leading to passive accumulation in certain tissues. The small size may also ease further penetration of the micellar carrier to cells (Lavasanifar, Samuel & Kwon, 2002). Their optimal critical micelle concentration value is usually in a low millimolar region or lower, and the loading efficiency towards hydrophobic drug should be between 5 and 25% wt (Torchilin, 2004).

1.1 Micellar Solubilization

Poor aqueous solubility is a big obstacle in drug development. It poses such a serious problem that some pharmaceutical companies choose to exclude poorly soluble compounds very early in their screening process regardless of how active these compounds are. It has been estimated that about 40% of potentially valuable drug candidates, including those with the highest activities, are rejected and never enter a formulation development stage due to their poor aqueous solubility (Lukyanov & Torchilin, 2004).

It was estimated that in the year 1999 the global retail sales of poorly soluble drugs were approximately 37 billion dollar, even though these compounds did not meet their full market potential due to formulation problems (Croy & Kwon, 2006).

Nowadays, various strategies are used to increase the solubility of drugs in order to increase their bioavailability, e.g. formation of a corresponding salt, use of surfactants such as polyethoxylated castor oil and use of organic solvents such as ethanol (Hillery, Lloyd & Swarbrick, 2001). A typical formulation of paclitaxel is a good example of the use of an organic solvent and a surfactant simultaneously. Paclitaxel has shown a high potential as an anticancer drug, however it has a poor aqueous solubility (approx. 1μg/mL) (S. C. Lee, Kim, Kwon, Chung & Jeong, 2003). For this reason paclitaxel is dissolved in the vehicle of 50% ethanol and 50% Cremophore EL (polyethoxylated castor oil) (Chabner et al., 2006). Number of studies have reported that Cremophore EL, which is biologically active, induces serious side effects such as hypersensitivity, hyperlipidemia, neurotoxicity, nephrotoxicity and the extraction of plasticizers from intravenous infusion lines (S. C. Lee et al., 2003). Szebeni et al. (1998) showed that Cremophore EL in the formulation of paclitaxel in Taxol® causes flushing in 40% of patients and hypersensitivity in 1.5 to 3% of patients. Polysorbate 80 is another biologically active surfactant and has been used in the pharmaceutical industry. It has been implicated as the cause of toxicities that are associated with an unusual syndrome and fatalities among infants which caused the intravenous vitamin E formulation E-Ferol® to be withdrawn from the market in 1984 (Alade, Brown & Paquet, 1986). Furthermore, the administration of many other co-solvents and surfactants causes toxic or other undesirable side effects (Torchilin, 2004). In this regard, the development of new vehicles that are safe for human administration is of great importance.

More recent approaches to increase solubility involve the use of a range of carrier systems, including liposomes, cyclodextrins, micromolecules and micelles (Torchilin, 2004) .

Micellar solubilization is a powerful alternative for dissolving poorly soluble drugs and is one of the most important properties of micelles. The ability of micelles to increase the solubility of sparingly soluble substances in water has a particular significance in pharmacy (Seedher & Kanojia, 2008). Micellar solubilization can be defined as: "*the spontaneous dissolving of a substance by reversible interaction with the micelles of a surfactant in a solvent to form a thermodynamically stable isotropic solution with reduced thermodynamic activity of the solubilized material*" (Rosen, 2004). The important property of the phenomenon from a practical point of view is that it dissolves substances in solvents in which they are normally insoluble. Solubilization into aqueous media is of major practical importance in such areas as the formulation of water insoluble drugs, where it can replace the use of organic solvents or co-solvents (Rosen, 2004).

It has repeatedly been shown that polymeric micelles can increase the solubility of poor aqueous soluble drugs. Velluto et al. explored the drug encapsulation of Ciclosporin A (CsA) into a block copolymer composed of poly(ethylene glycol) as the hydrophilic part and a poly(propylene sulphide) as the hydrophobic part. Considering that only 23 μg of CsA will dissolve in 1 mL of water, their results show an effective solubility increase of almost a 1000 fold at a polymer concentration of 10mg/mL (Velluto, Demurtas & Hubbell, 2008).

1.1.1 The Biopharmaceutics Classification System (BCS)

The FDA's Biopharmaceutics Classification System (BCS) is a scientific framework for classifying drug substances based upon their aqueous solubility by predicting *in vivo* performance of drug products from *in vitro* measurements of permeability and solubility (Amidon, Lennernäs, Shah & Crison, 1995).

According to BCS, a drug substance is considered "highly soluble" when the highest dose strength is soluble in ≤ 250 mL of aqueous media over the pH range of 1 to 7.5 and a drug substance is considered "highly permeable" when the extent of absorption in humans is determined to be ≥90 % of an administered dose based on a mass balance

determination or in comparison to an intravenous reference dose. In this classification a drug product is considered "rapidly dissolving" when ≥85% of the labelled amount of the substance dissolves within 30 minutes over the entire physiological range (e.g. at pH 1.3, 4.5 and 6.8 at 37°C) (Food and Drug Administration, 2000)

In the BCS drug substances are classified into four groups according to their solubility and permeability factors (Table 1) (Amidon et al., 1995):

Table 1. The Biopharmaceutics Classification System (BCS) (Amidon et al., 1995).

Class	Solubility	Permeability
I	High	High
II	Low	High
III	High	Low
IV	Low	Low

Mr. Leslie Benet and his team recently investigated the distribution of drugs on the market vs. small molecule new molecular entities (NME). The results of their research are listed in Table 2 (L. Benet, personal communication, March 27, 2009)

Table 2: Distribution of drugs on the market (603 oral drugs with immediate release) vs. small new molecular entities (L. Benet, personal communication, March 27, 2009).

Class	Marketed drugs	NMEs
I	41%	5%
II	32%	70%
III	22%	5%
IV	5%	20%

As one can see, there are many drugs in Class II to Class IV which underlines the importance of improving the dissolution of poorly soluble drugs.

1.2 Surfactants and micelles

Surfactants play a vital role in contemporary pharmaceutical biotechnology, since they are largely utilized in various drug dosage forms to control wetting, stability and bioavailability among other properties (Rangel-Yagui, Pessoa & Tavares, 2005).
The surfactants considered for drug delivery are characterized by at least two distinct segments, a hydrophilic or non-polar moiety known as the "head" and a hydrophobic or polar moiety known as the "tail". With distinctly different properties between the two segments, in aqueous solution the hydrophobic segment of the unimer will be poorly soluble while the hydrophilic segment is well soluble. A molecule that both has hydrophilic and hydrophobic segments in its structure is called an amphiphile. The surfactant tail is usually saturated or unsaturated hydrocarbon chains or, less commonly, heterocyclic or aromatic ring systems. The surfactant head is usually a long chain hydrocarbon residue that can be anionic, cationic, zwitterionic, or non-ionic (Florence & Attwood, 2006).

1.2.1 Critical micelle concentration

In 1913 McBain proposed a novel idea that molecules come together at a critical concentration to form aggregates. At that time this concept was quite novel but today it has gained universal acceptance. As the surfactant concentration is increased the surface tension decreases. This happens as the concentration of surfactant molecules at the surface increases. At a certain concentration of surfactant molecules, saturation is achieved and thus surface tension can not decrease further. When this concentration is reached, other mechanisms start in the solution in order to shield the hydrophobic part of the amphiphile from the aqueous surrounding. This happens through the formation of small spherical aggregates in the solution made of the surfactant. These aggregates are better known as micelles (Florence & Attwood, 2006).
When surfactants are present in an aqueous solution at a certain concentration they seek out to the surface and orientate themselves to form micelles. In micelles, the hydrophobic tails flock to the interior in order to minimize their contact with water, while the hydrophilic heads remain on the outer surface in order to maximize their contact with water and hence achieve a minimum free energy state (Florence &

Attwood, 2006; Rangel-Yagui et al., 2005). The heads of the micelles, exposed into the aqueous surrounding, consists of components that are hardly reactive towards blood or tissue components. This unique structure allows micelles to stay in the blood for rather a long time without being recognized by certain proteins and/or phagocytes. This longevity is an extremely important factor of micelles (Torchilin, 2001).

The hydrophobic groups (i.e. the tails) of the surfactant form the core of the micelles and are protected from the contact with water by the hydrophilic groups, which form a shell or a corona around them. In the presence of micelles, the concentration of surfactant molecules in the surface layer remains approximately constant and hence the surface tension (γ) γ-log concentration plot practically becomes horizontal. The concentration at which the micelles first form in a solution is called the *critical micelle concentration* (CMC). The CMC concentration coincides with the concentration at which there is a sudden change in the slope of the plot (Figure 1) (Florence & Attwood, 2006).

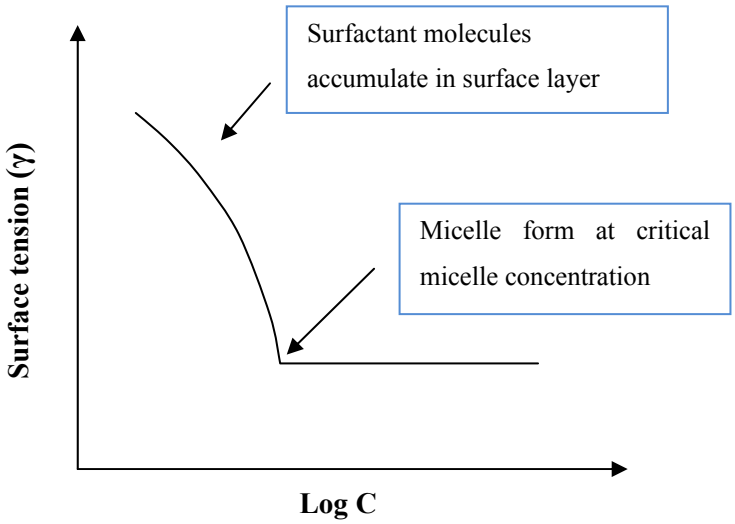


Figure 1. The critical micelle concentration (CMC). Plot of the surface tension (γ), vs. the logarithm of surfactant concentration (C). The CMC is reached where the slope becomes almost horizontal (this is a simplified graph).

Micellization occurs as a result of two forces. One is an attractive force that leads to the association of molecules while the other one, a repulsive force, prevents unlimited growth of the micelles to a distinct macroscopic phase. Below the CMC the molecules exist only as monomers in dispersion (Croy & Kwon, 2006).

Micellization (Figure 3) is driven entropically by the virtue of the hydrophobic effect. The hydrophobic effect describes the increase in entropy due to the increased disorder of water molecules that are no longer organized to minimize exposure to the hydrophobic segment of the surfactants. The water molecules are expelled from the core of the micelle. Micellization can be described in terms of free energy and the CMC by the following equation:

$$\Delta G^\circ = RT \ln(CMC)$$

Where ΔG° is the standard free energy change, R is the gas constant, and T is the Kelvin temperature of the system (Croy & Kwon, 2006).
The lower the CMC value is of a given amphiphilic polymer, the more stable micelles it forms. From a pharmacological point of view, this parameter is an important indicator of the stability of a micelle, since upon dilution with a large volume of the blood, considering intravenous administration, only micelles of surfactants with a low CMC value still exists, while micelles from surfactants with high CMC value may dissociate into monomers and their content may precipitate in blood (Rangel-Yagui et al., 2005).

There are many factors that influence the CMC. They include:

i. the properties of the hydrophobic segment
ii. the properties of the hydrophilic segment
iii. molecular weight
iv. temperature

The properties of the hydrophobic segment have the most profound effect on the CMC. By increasing its hydrophobicity and size the CMC decreases (Croy & Kwon, 2006). The effect of the length of the hydrophobic part on CMC has been investigated by Batrakova et al. (1999). In their study they examined poloxamers, where the hydrophobic block was poly(propylene oxide) (Figure 2). To see the effect of the hydrophobic block length, they increased the size of the poly(propylene oxide) block from approximately 15 repeating units to 70 (Batrakova et al., 1999).

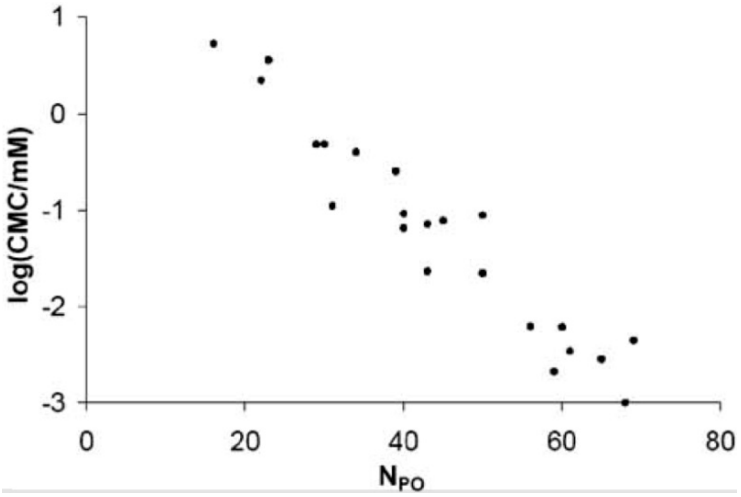


Figure 2. By increasing the hydrophobic segment length the critical micelle concentration (CMC) decreases. Effect of the number of poly(propylene oxid) repeating units (N_{PO}) on the CMC of poloxamer block copolymers (Batrakova et al., 1999).

By increasing the length of poly(propylene oxide) the CMC decreased, with high correlation. An increase in the hydrophilic segment has a contrary effect, i.e. by increasing the length of the hydrophilic segment the CMC increases (Croy & Kwon, 2006). The effect of changes in the hydrophilic segment size is much less than for changes of the hydrophobic segment. Thus the hydrophobic block is mainly responsible for the micellization.

When the ratio of the two segments is held constant and the molecular weight is increased the CMC value decreases. Temperature has an insignificant effect on the CMCs of non-ionic block copolymer micelles, with the exception of the poloxamers (Croy & Kwon, 2006).

Additional compounds can also affect the CMC value. Some highly hydrophobic compounds, such as fenofibrate (FF), incorporate into the cores of micelles and serve to increase the hydrophobicity of the core and drive the CMC value lower (Croy & Kwon, 2006). When an additive compound is used in CMC measurement, it is sometimes referred to as the critical association constant or CAC and when the CMC is measured in absence of a drug i.e. only surfactant with water it is referred as the CMC value (K. Y. Lee, Kwon, Kim, Jo & Jeong, 1998; Ye et al., 2008). In further references in this paper CAC will be used when the CMC is measured in a presence of an additive. The fact that a hydrophobic compound such as FF can lower the CMC is of great importance when it comes to using drug micellar systems as drug delivery systems.

Many methods can be used to measure the CAC and CMC. For CMC surface tension is often used. Fluorescence probing technique is currently the most sensitive technique to determine CAC and hence the most commonly used (Croy & Kwon, 2006).

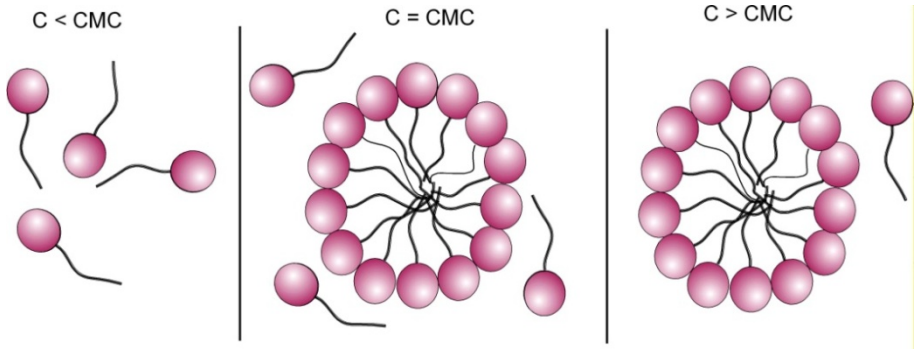


Figure 3. Schematic presentation of the micellization process. When C < CMC only monomers exists, when C = CMC, both micelles and monomers exists and when C > CMC micelles exists along with few monomers.

1.2.2 Micelle stability

Physical stability is fundamental for a micelle drug delivery system to withstand dissociation after entering the blood stream (Gaucher et al., 2005).
The stability of block copolymer micelles is often divided into two different categories; thermodynamic stability and kinetic stability. (Allen, Maysinger & Eisenberg, 1999).

1.2.2.1 Thermodynamic stability

The ability of micelles to withstand dilution is described in terms of their thermodynamic stability (Croy & Kwon, 2006). Micelle drug delivery systems are subject to severe dilution upon intravenous injection into animals and humans. In an average individual the blood volume is about 5 L. This means that an intravenous injection of a 2% (w/w) PCL_{21}-β-PEG_{44} micelle solution, will have a concentration of copolymer in the blood of only 400 mg/L (Allen et al., 1999). As described above, micelles assemble at the CMC or CAC. Conversely, these values also describe the magnitude to which a micelle can withstand dilution. Micelles with the lowest CMC/CAC can withstand the most dilution, and hence are the most thermodynamically stable. When the concentration of an amphiphile decreases below the CMC/CAC in a solution, the micelles disassociate into unimers to re-establish the thermodynamic equilibrium (Croy & Kwon, 2006).

1.2.2.2 Kinetic stability

As described above, micelles should disintegrate upon dilution below the CMC/CAC. In theory this is true, but one must not forget the timescale of disassembly. The timescale of micellar disassociation depends on the kinetic stability of micelles (Croy & Kwon, 2006).

The disassembly of block copolymer micelles at a copolymer concentration below the CMC or CAC has been reported to be quite slow (Allen et al., 1999). There are several factors that influence the kinetic stability of micelles. These include; viscosity of the core, glass transition temperature (T_g) and core size (Croy & Kwon, 2006).

Micelles formed from a block copolymer that has a high T_g are described as "frozen" where segmental motion and disassociation will tend to disassemble appreciably slow and enable the maintenance of an intact micelle for some time after dilution below the CMC/CAC. These micelle cores are described as solid-like, while micelle cores with a low T_g are described as liquid-like and will disassociate rapidly upon dilution (Croy & Kwon, 2006).

Below the CMC/CAC only single chains exist but above it both micelles and single chains exist. Nonetheless, micelles may be kinetically stable below the CMC/CAC and exist at least for some period of time (Lavasanifar et al., 2002). Incorporation of hydrophobic compounds into block copolymer micelles can enhance micellar stability. Yokoyma et al. (1998) showed that by incorporating adriamycin into block copolymer micelles by both physical entrapment and by chemical conjugation increased the structural stability of the micelles. They suggested that the presence of a hydrophobic drug increased the hydrophobic interaction and produced micelles that were more tightly packed.

Strong cohesive forces between a drug and a polymer core segment can add physical or kinetic stability to the system. As a result, a slow dissociation rate may exist for polymeric micelles below the CAC, and polymeric micelles may not necessarily exist in equilibrium with polymeric unimers. Kinetic stability may be high for polymeric micelles with stiff or bulky core-forming blocks due to hindrance of rotation (Lavasanifar et al., 2002).

The various factors influencing micellar stability are listed in Table 3.

Table 3. The various factors which influence the stability of block copolymer micelles.

Parameter		Micellar stabiliy
CMC	Low	↑
	High	↓
T_g	Low	↓
	High	↑
Hydrophobic drug content	Low	↓
	High	↑

1.2.3 Locus of solubilization

Micelles made of non-ionic surfactants (the most frequently used in micelle formation) are known to have an anisotropic water distribution within their structure, i.e. the water concentration decreases from the surface towards the core of the micelle, with a completely hydrophobic core. Consequently, the spatial position of a solubilizied drug in a micelle will depend on its polarity (Torchilin, 2001).

In aqueous systems there are a number of possible loci of solublization for a drug in a micelle (Figure 4).

i. Nonpolar molecules will be solubilizied in the core of the micelle
ii. Substances with intermediate polarity will be distributed along surfactant molecules in certain intermediate position
iii. Polar molecules will be absorbed on the micelle surface (Torchilin, 2001).

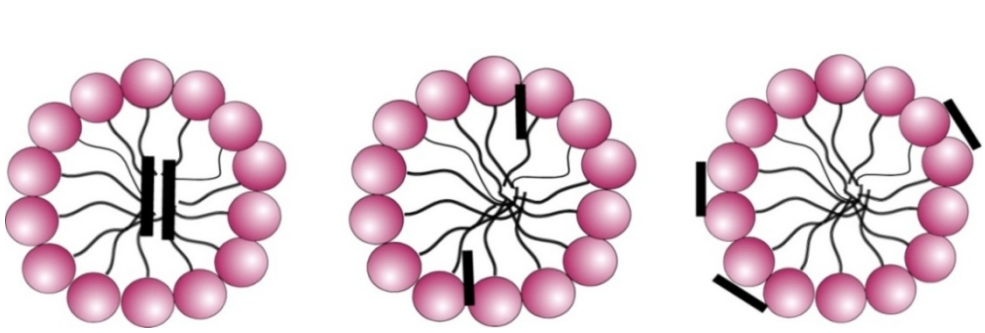


Figure 4. Possible loci of solubilization of drugs in micelles, depending on the drug hydrophobicity. The black bold lines represent the drug at different sites of the micelle. The pink bulbs represent the surfactant heads, and the black curved lines represent the surfactant tails.

The existence of different sites of solubilization in/on the micelle are due to the fact that the physical properties, such as microviscosity, polarity and hydration degree, are not uniform along the micelle (Rangel-Yagui et al., 2005).

1.3 Polymeric micelles for drug delivery

Polymeric micelles are formed by block copolymers that self-assemble into micelles in water. A polymeric micelle usually consists of several hundred copolymers and has a diameter of 10 to 100 nm (Kwon & Okano, 1996).

Compared to surfactant micelles, polymeric micelles are more stable; with a much lower CMC/CAC. Polymeric micelles have a slower rate of dissociation, allowing retention of loaded drugs for a longer period of time (Kataoka, Kwon, Yokoyama, Okano & Sakurai, 1993). Contrary to liposomes that have been shown to incorporate water-insoluble drugs into the aqueous interior, polymeric micelles can be vehicles for hydrophobic and sparingly soluble pharmaceuticals. Unlike liposomes, polymeric micelle do not need to compete with any naturally occurring particles or molecules for receptor binding (Torchilin, 2001). In addition, some polymeric micelles present better solubilization capacity when compared to surfactant micelles due to the higher number of micelles and/or larger cores of the formers (Rangel-Yagui et al., 2005).

1.3.1 Block copolymers

Several classifications of polymers and copolymers occur. For one to understand the basics of such copolymers a very simple classification is represented in Figure 5.

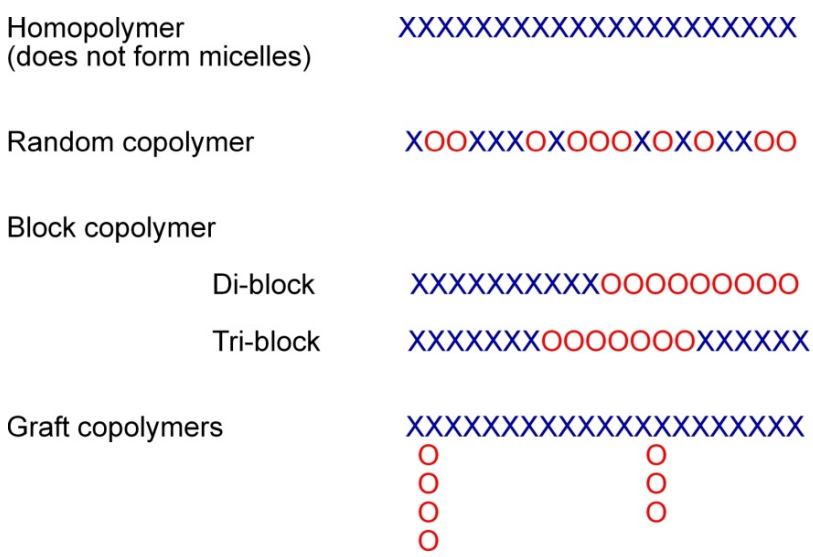


Figure 5. Main structural types of copolymers. X represents the hydrophilic part and O the hydrophobic part.

Micelles formed from di- and triblock-copolymers are of particular interest in pharmaceutics. Unlike homopolymers built of identical monomers, copolymers include 2 types of monomers that differ in solubilization. Those two types of monomeric units can be built up in a different way to form random, block or grafted copolymers (Torchilin, 2001).

Types of micelles formed from different block copolymers are shown in Figure 6.

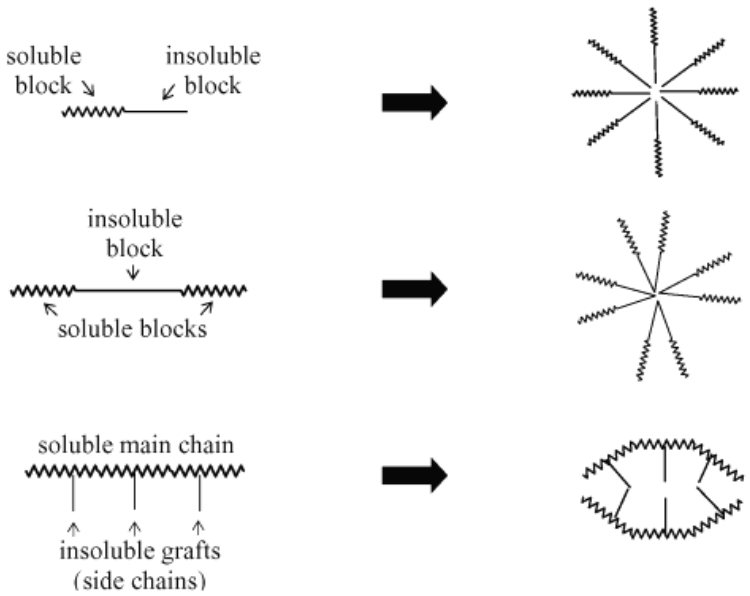


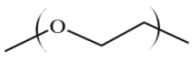
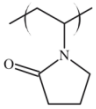
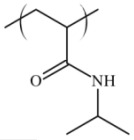
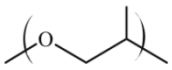
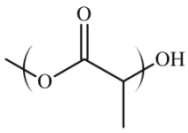
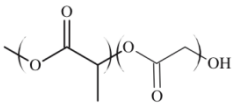
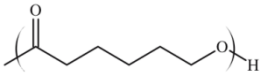
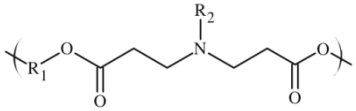
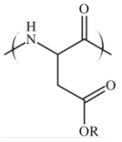
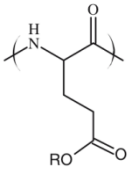
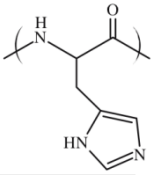
Figure 6. Mechanism of micelle formation from different types of amphilic copolymers (Rangel-Yagui et al., 2005).

Many types of copolymers have been used for micelle formation. The requirement for safe administration regarding biocompatibility and frequently biodegradability has limited the choice of copolymers (Sutton, Nasongkla, Blanco & Gao, 2007). For the hydrophilic block of these systems, poly(ethylene glycol) (PEG), also known as poly(ethylene oxide) (PEO) depending on the manufacturer, is the most commonly used, with a molecular weight ranging from 285 to 8,000,000 g/mol (Sigma-Aldrich, 2009). PEG is completely water soluble, non toxic and uncharged, the latter property serving to lessen the possibility of interaction with plasma proteins. PEG has been used safely in humans and is approved by regulatory agencies for administration. The use of other hydrophilic polymers as shell-forming blocks has been reported for bioadhesive or thermoresponsive properties (Lavasanifar et al., 2002; Sutton et al., 2007).

Other hydrophilic polymers such as poly(N-vinyl pyrrolidone) (PVP) and poly(N-isopropyl acrylamide) have been used to form the corona layer of micelles (Sutton et al., 2007). Unlike the corona-forming block, the choice for a core-forming block is relatively diverse. The length of the core-forming block is usually equal or shorter than the PEG block to maintain water solubility and form spherical core/shell micelle structures (Lavasanifar et al., 2002). The most common materials in core-forming blocks are hydrophobic polyesters but other materials, such as polyethers, polypeptides and poly(ß-amino esters) have also been used. Both polyesters and polyamides can undergo hydrolytic and enzyme-catalyzed degradations and are considered biodegradable (Sutton et al., 2007). One of the most commonly used block copolymer is a poly(ester) named poly(ethylene glycol)-block-poly(ε-caprolactone) (PEG-β-PCL). It has been shown that micelles made from PEG-β-PCL form nano-sized micelles with a drug loading efficiency up to 95% and a low CMC (Jette, Law, Schmitt & Kwon, 2004). Sheikh et al. (2009) have shown that Poly(ε-caprolactone)-grafted-poly(vinyl alcol) (PCL-g-PVA) block copolymers self-assemble into nanocarriers and can be used for both hydrophobic and hydrophilic drugs. Furthermore, the PCL-g-PVA drug-loaded micelles revealed continuous and sustained release for up to 20 days. Another group designed PEG-β-PCL micelles for targeted delivery for cancer treatment. The nano-sized micelles had a drug loading efficiency of paclitaxel up to 54.3% and the micelles showed sustained release. The micelles were selectively taken into cancer cells by endocytosis, hence showed higher toxicity for cancer cells than other cells.

In the late 1980s and the early 1990s Kataoka and his team in Tokyo synthesized di-block copolymers. At the same time, and independent of Kataoka et al.'s work, Kabanow developed drug loaded PEGylated micelles based on PEG-PPO-PEG tri-block copolymers known as Pluronics® (Hoffman, 2008). Many studies on block copolymers have been conducted on Pluronics®. Pluronics® demonstrate solubilizing effects for parenteral drug administration. Pluronics® have been used to solubilize many drugs such as; haloperidol, indometacin, doxorubicin, epirubuxin and amphotericin B. Overall, many Pluronics® used for drug solubilization have high ratios of PEG to PPO and are non-toxic relative to many low molecular weight surfactants, such as Tween 80, especially in terms of cell membrane lysis, e.g. haemolysis (Lavasanifar et al., 2002). Commonly used block polymer segments are listed in Table 4.

Table 4. Commonly used block segments of copolymers used in micellar drug delivery systems (Sutton et al., 2007).

Copolymers	Abbreviation	Repeating Unit Structure
Corona segment		
Poly(ethylene glycol) / Poly(ethylene oxide)	PEG/PEO	
Poly(N-vinyl pyrrolidone)	PVP	
Poly(N-isopropyl acrylamide)	NIPAM	
Core segment		
Poly ethers		
Poly(propylene oxide)	PPO	
Polyesters		
Poly(L-lactide)	PLA, PDDLA*	
Poly(D-lactide)		
Poly(lactide-co-glycolide)	PLGA	
Poly(ε-caprolactone)	PCL	
Poly(β-amino ester)		
Polyamides		
Poly(L-aspartic acid) derivatives	pAsp	
Poly(L-glutamic acid) derivatives	pGlu	
Poly(L-histidine)	pHis	

1.3.2 Micellar size

The most important property of micelles is their "ideal" small size. Their size range is usually between 10 to 100 nanometers (nm), and is similar to that of viruses (Kataoka, Harada & Nagasaki, 2001). The size of the polymeric micelles not only makes them ideal drug delivery carriers as they escape from renal exclusion in the reticulo-endothelial system but also enhances their vascular permeability (Hu, Ren, Yuan, Du & Zeng, 2006).

The size of micelles is primarily dependent on the length of the hydrophobic and hydrophilic segments. The size of the core is dependent on the length of the hydropobic segment. The molecular weight and relative proportion of hydrophobic and hydrophilic blocks also affects the size of polymeric micelles (Croy & Kwon, 2006). Micellar size can be obtained directly by dynamic light scattering (DLS). DLS is a popular method to determine particle size due to its sensitivity (approx. 3 nm) and conveniency (Croy & Kwon, 2006).

1.3.3 Micelle preparation

The choice of method used for micelle preparation depends mostly on the solubility of the block copolymer in water (Allen et al., 1999).

Commonly used methods to prepare micelle solutions and hence entrap a drug into the micelles are:

i. direct dissolution
ii. solvent evaporation
iii. co-solvent evaporation
iv. dialysis

Direct dissolution is the simplest method and can only be used for highly soluble surfactants that form micelles upon aqueous dissolution. The other methods allow for dissolution of drug and polymer in an organic solvent with removal of the organic solvent with either evaporation or slow replacement of water through a semi-permable membrane with a molecular weight cut off (MWCO).

1.3.4 Micellar morphology

Recently, researchers have investigated the morphologies of the assemblies formed by amphiphilic block copolymers. In general, micelles formed by amphiphilic copolymers are reported to be spherical micelles (Bronstein et al., 2006; L. Chen, Xie, Hu, Chen & Jing, 2007; Letchford, Liggins, Wasan & Burt, 2009; Li, Dai, Zhang & Zhuo, 2008; Siddiqui, Kumar & Kabir-ud-Din, 2009; Velluto et al., 2008)

However, as has been shown in numerous studies from Eisenberger and his group, block copolymer aggregates can exist in many different morphologies, such as, spheres, starfish-shape, rods, various vesicles, needles, tubules, lamellae and many more. (Choucair & Eisenberg, 2003a, 2003b; Gao, Varshney, Wong & Eisenberg, 1994; Yu & Eisenberg, 1998).

Furthermore, the spherical core represents a molten liquid globule and a swollen corona surrounding its core. The liquid nature of the core permits effective mixing of hydrophobic blocks that leads to a thermodynamic equilibrium state. This is a quasi-equilibrium since a simple dilution can shift micelle dissociation.

However, certain micelles dissociate very slowly into monomers that demonstrate considerably high kinetic stability. At a concentration above the CMC micelles can exist for an indefinitely long period, so their storage stability should not be an issue (Torchilin, 2001).

1.3.5 Drug loading and entrapment efficiency of polymeric micelles

The micelle core serves as a nanoreservoir for various hydrophobic molecules (Lavasanifar et al., 2002). The mechanism of drug loading into micelles is generally categorized into two groups:

i. chemical encapsulation
ii. physical encapsulation

In chemical encapsulation the drug is chemically conjugated on the core-forming block of the copolymer. This method is mostly used when a drug is intended in drug targeting or when the release is supposed to be over an expended period of time. With chemical conjugation higher drug loading is achieved and it minimizes the likelihood that the drug will be prematurely released from the micelle (Croy & Kwon, 2006). Normally,

the drug will be inactivated while bound to the surfactant, which is an extremely good option for toxic drugs such as cancer drugs.

Physical encapsulation is simpler than conjugated encapsulation as there is no need for chemical synthesis. The drug selectively partitions into the core i.e. the cargo space of the micelle (Croy & Kwon, 2006).

However, this cargo space is limited: for instance, a typical 1% (w/w) PCL-β-PEG micelle solution contains approximately 0.5 % core volume (Allen, Yu, Maysinger & Eisenberg, 1998). This means that in a 10 mL sample of this 1% (w/w) micellar solution, the core volume is only 50 µL.

The extent of incorporation into block copolymer micelles by physical means is dependent on several factors, including the molecular volume of the solubilizate, its interfacial tension against water, polarity, hydrophobicity, length of the core and shell-forming blocks in the copolymer, and the polymer and solubilizate concentration. The partition coefficient of the hydrophobic molecule between the micellar core and surrounding aqueous medium describes the extent of drug entrapment in polymeric micelles (Allen et al., 1999; Lavasanifar et al., 2002).

The nature of the solute, including polarity, hydrophobicity, charge and degree of ionization has also been found to influence the incorporation in the micellar core. However this is entirely dependent on the nature of the core-forming block. It is the compatibility between the solute and the coreforming block that can be used to enhance incorporation most effectively (Allen et al., 1999) .

The greatest degree of solubilization occurs when high compatibility exists between the micellar core and the solubilizate, described by the Flory-Higgins interaction parameter (χ_{sp}).

$$\chi_{sp} = (\delta_s - \delta_p)^2 V_s/RT$$

Where χ_{sp} = interaction parameter between the solubilizator (s) and the core-forming polymer block (p), δ_s = the Scatchard- Hilderbrand solubility parameter of the solubilizate, δ_p = the Scatchard-Hilderbrand solubility parameter of the core-forming polymer block and V_s = the molar volume of the solubilizate, R is the gas constant and T the Kelvin temperature (Allen et al., 1999).

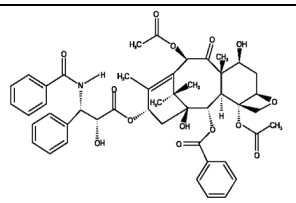
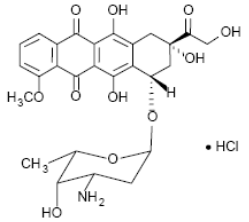
The lower the positive value of the interaction parameter (χ_{sp}) the greater the compatibility between the solubilizate and the core-forming block. The highest degree of compatibility is achieved when $\delta_s = \delta_p$ (Lavasanifar et al., 2002).

The complex interaction between the polymer and the drug suggests that the largest amount of drug loaded per micelle will be reached when the core-forming block is most suitable for a specific drug. Since each drug is unique, no single micelle forming block will enable maximum loading levels for all drugs. Hence, no micellar system will be viable as a universal delivery vehicle for all hydrophobic drugs (Allen et al., 1999).

Two of the most investigated drugs for delivery in block copolymer micelles are paclitaxel and doxorubicin. Doxorubicin is an amphiphilic compound that has logP of 0.52 and aqueous solubility of 50 mg/mL (Liu, Lee & Allen, 2006). Paclitaxel is a highly hydrophobic drug with logP of 3.96 and a aqueous solubility of 1µg/mL (Liu et al., 2006). Table 5 shows examples of copolymers used for incorporating doxorubicin and paclitaxel, their drug loading and their physicochemical properties.

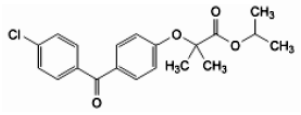
The drug fenofibrate is often used as a model of a hydrophobic drug. It belongs to class II (poor solubility and high permeability) of the BCS system (Table 1). FF is practically insoluble in water (solubility is reported from 0.1µg/mL to 0.8 µg/mL) (S. Jamzad & R. Fassihi, 2006; Jette et al., 2004; SRC PhysProp Database, 2009; Vogt, Kunath & Dressman, 2008). Table 6 summarizes the entrapment efficiency of copolymers used to entrap FF and the physicochemical properties of FF.

Table 5. Summary of the physicochemical properties of doxorubicin (DOX) and paclitaxel (PTX), the copolymers employed for formulation of these drugs and the drug loading levels (Liu et al., 2006).

Structure	Log P[a]	Aqueous Solubility	Copolymers	Loading Level (wt/wt)
Paclitaxel 	3.96	1µg/mL	Poly(N-vinylpyrrolidine)-β-poly(D,L-lactade)	5 %
			Poly(ethylene glycol)-β-poly(D,L-lactide)	25 %
			Poly(ethylene glycol)-β-poly(D,L-lactide)	5.1 %
Doxorubicin 	0.52	50 mg/mL	Poly(etylene glycol)-β-poly(DL-lactid-co-glycolic acid)	19.2 %
			Poly(ethylene oxide)-β-poly(β-benzyl-L-aspartate)	20 %
			Poly(ethylene glycol)-β-poly(ε-caprolactone)	4.3 %
			Poly(etylene glycol)-β-poly(aspartic acid)	7.3 %

[a]Partition coefficient of the solute

Table 6. Summary of the physicochemical properties of fenofibrate, the polymers employed for formulation of the drug and the entrapment efficiency (Jette et al., 2004; Klose et al., 2009; Sant, Smith & Leroux, 2005; SRC PhysProp Database, 2009).

Structure	Log P[a]	Aqueous Solubility	Copolymer	Entrapment Efficiency
Fenofibrate 	5.19	0.1µg/mL to 0.8µg/mL	Poly(vinyl alcohol)-β-Poly(D,L lactide-co-glycolide acide)	25.7 %
			Poly(ethylene glycol)- Poly(ε-caprolactone)	95 %
			Poly(etylene glycol)-β-poly(alkylate-co- methacrylic acid)	75 %

[a]Partition coefficient of the solute

1.3.6 Drug release

A significant advantage of micelles is their potential of sustained drug release. Their ability to achieve slow drug release is dependent on several factors. First of all the micelle has to be stable when diluted. That means, as has been mentioned before, that the micelle must be thermodynamically and kinetically stable. Moreover, if the drug is enclosed in the micellar core, the micelle must be able to prevent fast diffusion of the

active molecule from the core into the biological surroundings. Drugs that are physically encapsulated in a micelle require extremely small diffusion coefficients (approx. 10^{-16} to 10^{-18}cm^2/sec) to qualify for standard release, depending on the core and the probe (Teng, Morrison, Munk, Webber & Prochazka, 1998).
Despite the fact that some researchers have reported that micelles have sustained release, fast drug release from micelles is the biggest disadvantage of micelles as a drug delivery system (Hu, Ren et al., 2006; Kang, Kim, Han & Chang, 2002; Ye et al., 2008).

1.3.7 The effect of stearic acid on micelle stability

Several strategies have been used to maximize the drug loading efficiency and to control the drug release rate of micelles. One of the strategies to enhance the stability of micelles is the use of stearic acid (SA) (Hu, Ren et al., 2006; Hu, Zhao et al., 2006; Ye et al., 2008; Zhang, Yie, Li, Yang & Nagai, 2000)
SA is an endogenous long-chain (C_{18}) saturated fatty acid (Figure 7) and one of the main components of triacyl glycerols (bodyfat). SA is biocompatible with low toxicity and hence, is accepted for pharmaceutical use (Hu, Zhao et al., 2006; Zhang et al., 2000). SA is in solid state at room temperature end therefore it is supposed to stabilize micelles. SA is available for pharmaceutical use and it is easy to use in the preparation of nanoparticles (Zhang et al., 2000).

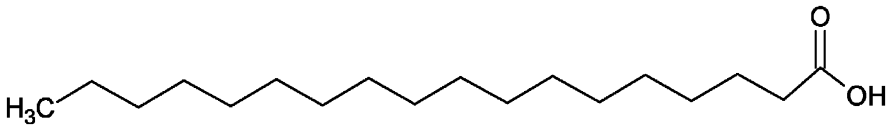


Figure 7. Stearic acid, a long chain saturated fatty acid.

In order to increase drug and gene loading, and to control the release rate in chitosan oligosaccharide (CSO) micelles, Hu et al. (2006) grafted stearic acid to amino groups of the CSO, forming stearic acid grafted chitosan oligosaccharide micelles (CSO-SA) with different degree of amino substitution (SD). They observed, in several studies, that the solubility of micelles enhanced with the SD of CSO-SA and the release rate of drug

from the micelles was lowered by increasing the SD of CSO-SA (Hu, Li & Yuan, 2006; Hu, Zhao et al., 2006).

Furthermore, Hu and his team have shown that with increasing concentration of SA in micelles the particle size decreases. The reason for this size reduction is the increase of hydrophobic SA segments that results in enhanced interaction between the SA segment of the CSO-SA molecule (Hu, Ren et al., 2006).

1.3.8 Boc-(Leu-Leu-Ala-Lac)$_5$-Leu-Phe-O-PEG$_{4000}$-OH (DP7)

DP7 is a novel di-block copolymer synthesised by professor Katakai in Gunma University in Japan (Figure 8).

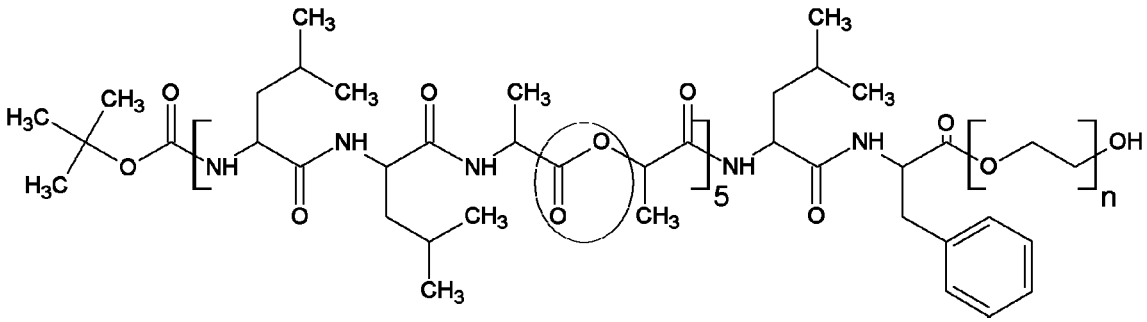


Figure 8. A chemical structure of DP7. The ester bond that replaces the peptide bond is in the ring and n (≈ 90) represents repeating units of poly(ethylene glycol) (PEG).

The hydrophobic part is a depsipeptide and the hydrophilic part consists of poly(ethylene glycol) units, more abbreviated PEG$_{4000}$ (Figure 9). Depsipeptide is made of a peptide that contains amino acids and hydroxyl acids in which one or more of the peptide bonds have been replaced by ester bonds (Katakai, Kobayashi, Yamada, Oku & Emori, 2004). DP7 contains three types of amino acids; Leucine (Leu), Alanine (Ala), and phenylalanine (Phe). In addition, DP7 contains lactic acid (Lac) that forms an ester bond instead of a peptide bond (as one can see in Figure 8, there are five ester bonds in each DP7 molecule). Tert-butoxycarbonyl (Boc) is an amino protecting group which is widely use in synthetic bioorganic chemistry (Pozdnev, 1974). The Boc-group contributes to the formation of a stable helix of protected hydrophobic linear depsipeptides such as Leu-Leu-Ala repeating units (Yamada, Sato, Oku & Katakai, 2003).

The molecular weight of DP7 is 6208.8 g/mol. There are no published data of DP7 and it has never before been investigated thoroughly (Y Makino, personal communication, April 4, 2009).

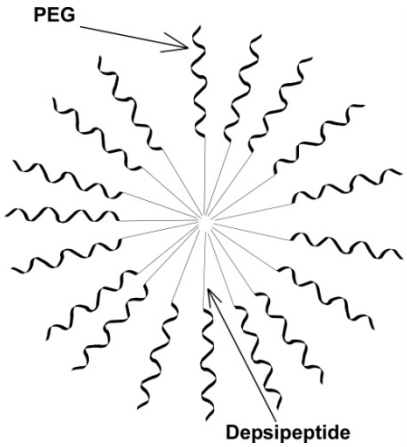


Figure 9. A micelle formed from DP7. The hydrophilic part is poly(ethylene glycol) and the hydrophobic part is a depsipeptide.

2. AIM

Micelles formed from amphiphilic block copolymers have been receiving much attention as nanocarriers in the field of drug delivery. Micellar solubilization is a powerful option for dissolving poorly soluble drugs. Boc-(Leu-Leu-Ala-Lac)$_5$-Leu-Phe-O-PEG$_{4000}$-OH or DP7 is a novel di-block copolymer that has never been studied thoroughly before.

The main aim of this study was to explore the solubilizing potential of DP7 micelles for poorly soluble drugs in order to improve their bioavailability. Another objective was to characterize the micelles and to evaluate if fatty acids, such as stearic acid, can improve the efficacy of the micelles.

Hence, the aim was divided into the six following steps:

1) To compare DP7 with commercial block copolymers and to analyse characteristics of DP7, such as:
    i. Glass transition temperature and melting point
    ii. Critical association concentration and critical micelle concentration
2) To evaluate which method is the most suitable for preparation of DP7 micelles.
3) To survey the effect of stearic acid on micelle efficacy.
4) To analyse characterization of micelles, such as:
    i. Particle size
    ii. Drug entrapment of fenofibrate
    iii. Particle distribution
5) To isolate ideal micelles from other particles in a micelle solution.
6) To evaluate drug release from fenofibrate loaded micelles.

3. MATERIALS, EQUIPMENT AND METHODS

3.1 Materials

3.1.1 Preparation of micelle solutions

3.1.1.1 Nanomizer method

- Boc-(Leu-Leu-Ala-Lac)$_5$-Leu-Phe-O-PEG$_{4000}$-OH (DP7) generously provided by Dr. Ryoichi Katagai at Gunma University, Maebashi City, Japan
- Ultra pure water (Milli-Q®)
- Stearic acid, Grade I, approx. 99% capillary GC. Sigma Aldrich, Germany. Lot: 027K5305
- Chloroform Japanese Industrial Standard (JIS), Guaranteed Reagent Grade (GRG). Wako, Osaka, Japan. Lot: DPL6005
- Poly (ethylene Oxide-β-caprolactone), Mn: PEO$_{5000}$-β-PCL$_{5000}$. Mw/Mn: 1.10. Sample# P7512-EOCL. Polymer Source, Inc.
- Poly (ethylene Oxide-β-caprolactone), Mn: PEO$_{5000}$-β-PCL$_{2300}$. Mw/Mn: 1.14. Sample# P3049-EOCL. Polymer Source, Inc.
- Poly (ethylene Oxide-β-caprolactone), Mn: PEO$_{5000}$-β-PCL$_{11000}$. Mw/Mn: 1.12. Sample# P8307-EOCL. Polymer Source, Inc.
- Poly (ethylene Oxide-β-caprolactone), Mn: PEO$_{5000}$-β-PCL$_{18000}$. Mw/Mn: 1.48. Sample# P-EOCL. Polymer Source, Inc.
- Fenofibrate, minimum 99%. Sigma Aldrich. Lot: 117K1486
- Cholesterol, JIS. GRG. Wako, Osaka, Japan. Lot: WKF0182

3.1.1.2 Co- solvent method

- Boc-(Leu-Leu-Ala-Lac)$_5$-Leu-Phe-O-PEG$_{4000}$-OH (DP7)
- Fenofibrate, minimum 99%. Sigma Aldrich. Lot: 117K1486
- Stearic acid, Grade I, approx. 99% capillary GC. Sigma Aldrich, Germany Lot: 027K5305
- Acetone JIS.GRG. Wako, Osaka, Japan. Lot: TFG0243

- Ultra pure water (Milli-Q®)

3.1.1.3 Thin film method

- Boc-(Leu-Leu-Ala-Lac)$_5$-Leu-Phe-O-PEG$_{4000}$-OH (DP7)
- Fenofibrate, minimum 99%. Sigma Aldrich. Lot: 117K1486
- Stearic acid, Grade I, approx. 99% capillary GC. Sigma Aldrich, Germany Lot: 027K5305
- Acetone. Wako, Osaka, Japan. Lot: TFG0243
- Poly (ethylene Oxide-β-caprolactone), Mn: PEO$_{5000}$-β-PCL$_{5000}$. Mw/Mn: 1.10. Sample# P7512-EOCL. Polymer Source, Inc.
- Ultra pure water (Milli-Q®)

3.1.2 Characteristics of micelles

3.1.2.1 Quantification of stearic acid

- Fatty Acid Assay Kit, Bio Vision. Cat.K612-100. Lot: 31112 (Fatty Acid Buffer, Fatty Acid Probe, Dimethylsulfoxide, ACS Reagent, Enzyme Mix, Enhancer, Palmitic acid standard)
- Dimethylsulfoxide (DMSO) JIS. GRG. Wako, Japan. Lot: TSH 9501
- Stearic acid, Grade I, approx. 99% capillary GC. Sigma Aldrich, Germany. Lot: 027K5305
- Polyoxyethylene (10) octophenyl esther, (Triton-X), JIS GRG. Wako, Japan Lot: ASR7871

3.1.2.2 Quantification of fenofibrate

- Fenofibrate, minimum 99%. Sigma Aldrich. Lot: 117K1486
- Acetonitrile. HPLC grade. Kanto Chemical Co., Inc. Tokyo, Japan Lot: 010 x 1694
- Ultra pure water (Milli-Q®)
- Carrier solution, ACN/H_2O (1/2)

3.1.2.3 Gel filtration chromatography

- Carrier solution, Phosphate buffer (pH 7.4)

3.1.2.4 CAC and CMC

- Pyrene, Aldrich. Recrystalized from EtOH twise (16.01.2007), by Yuji Makino
- Boc-(Leu-Leu-Ala-Lac)$_5$-Leu-Phe-O-PEG$_{4000}$-OH (DP7)
- Ultra pure water (Milli-Q®)

3.1.3 Separation of ideal micelles from other particles in micelle solution

3.1.3.1 Dialysis

- Aceton, JIS. GR grade. Wako, Osaka, Japan. Lot: TFG0243
- Acetonitrile, JIS. GR grade. Kanto Chemicalco., Inc. Lot: 010 x 1694
- Ultra pure water (Milli-Q®)

3.1.4 *In vitro* drug release studies from FF-loaded micelles

- Phosphate buffer solution (PBS), pH= 7.4,Nippon Gene Co., Ltd., Tokyo, Japan Lot: 00357H
- pH= 6.8, Nacalai Tesque, Inc. Kyoto, Japan. Lot: L8P1883
- pH= 1.2, Nacalai Tesque, Inc. Kyoto, Japan. Lot: L8P1882

3.1.5 T_g, T_m and degradation of DP7, PEG$_{5000}$-PCL$_{5000}$ and PEG$_{4000}$ / DSC analysis

- Boc-(Leu-Leu-Ala-Lac)$_5$-Leu-Phe-O-PEG$_{4000}$-OH (DP7)
- Polyethyleneglycol (PEG$_{4000}$), Merck, Lot: 807490

3.2 Equipment

3.2.1 Preparation of micelle solutions

3.2.1.1 Nanomizer method

- Homogenizer, Polytron® System PT 3100. Kinematica AG, Switzerland
- Glass jar with white cap, 50 mL, As One, Japan. Lot: 210105
- Wire
- Nanomizer®,YoshidaKikai Co.,Ltd. Japan. Ser. No. 29520140
- Cooler, Eyla CA-1112, China. Ser. No. 60716709
- Counter

3.2.1.2 Thin film method

- 100 mL recovery flask
- Evaporator, Eyela rotary evaporatot N-1000
- Cooler, Eyela CCA-1110. Ser.No. 60607644
- Water bath, Eyela SB-350
- 1000 mL round flask, Eyela
- Tube mixer trio, Tm-1F. As One Corporation, Osaka, Japan
- Ultra sonic bath, As One Corporation, Osaka, Japan

3.2.1.3 Co-solvent evaporation method

- 100 mL recovery flask
- Evaporator, Eyela rotary evaporatot N-1000, China
- Vacuum controller, Eyela, NTC- 2000, China
- Cooler, Eyela CCA-1110. Ser.No. 60607644. China
- Water bath, Eyela SB-350, China
- 1000 mL round flask, Eyela, China

3.2.2 Characteristics of micelles

3.2.2.1 Quantification of stearic acid

- Nunc™, Immuno plate, Thermo Fisher Scientific Inc, Denmark. Lot: 101841
- Cute Mixer, CM-1000. Ser No: 60606495
- Oven, Eyela WFO-500, China. Sr.No: 60606495
- Microcentrifuge tube 0,5 mL, No. ST-500. Ina Optica Co Ltd., Osaka, Japan
- Ultrasonic bath, As One Corporation, Osaka, Japan
- Tube mixer, As One Corporation, Osaka, Japan
- Lighter
- Microplate reader, model 680, Bio-Rad Laboratories. Inc.

3.2.2.2 Quantification of fenofibrate

- High-performance liquid chromatography (HPLC), Prominence®, Shimadzu Corporation, Japan
- Software, Lab solutions, Lc Solutions Version 1.22 SP 1, Shimadzu Corporation, Japan
- Capell Pak, C-18, type: UG 120, N=14976, Shiseido, Japan. Lot: H-18
- HPLC sample vial: Sample cup IA, Shinwa Chemical Industries, Ltd, Japan
- Pipette, Iwaki, IK-PAS-5P,Asahi techno glass, USA

3.2.2.3 Particle size

- Dynamic light scattering, Zetasizer Nano-ZS, Malvern Instruments Ltd., UK
- Software, Malvern Zetasizer, Malvern Instruments Ltd., UK
- Plastibrand®, UV-Cuvette micro, Wertheim, Germany
- Microcentrifuge tube, 5 mL, I No. ST-500, Ina Optica
- Pipette, Iwaki, IK-PAS-5P,Asahi techno glass, USA
- Paperholder for microcentrifuge tube

3.2.2.4 Gel filtration chromatography

- High-performance liquid chromatography (HPLC), Prominence®, Shimadzu Corporation, Japan
- Superose™ 6 10/300 GL, GE Healthcare Bio-Sciences AB, Sweden
  Lot: 1001543

3.2.2.5 CAC and CMC

- Fluorescence Spectrophotometer, Hitachi. Serial No: 1911-09
- Delta-8, Kibron Inc., Finland, Serial No. 811A050104019
- Nunc™, Immuno plate, Thermo Fisher Scientific Inc, Denmark. Lot: 101841

3.2.3 Separation of ideal micelles from other particles in micelle solution

3.2.3.1 Dialysis

- Spectra/Por® Biotech, regenerated cellulose dialysis membrane, MWCO: 3,500 Da. Spectrum Laboratories, Inc. Lot: 3228284
- Spectra/Por® Biotech, regenerated cellulose ester dialysis membrane, MWCO: 5,000 Da. Spectrum Laboratories, Inc. Lot: 3232292
- Spectra/Por® Biotech, regenerated cellulose dialysis membrane, MWCO: 8,000 Da. Spectrum Laboratories, Inc. Lot: 3230442
- Spectra/Por® Biotech, regenerated cellulose dialysis membrane, MWCO: 10,000 Da. Spectrum Laboratories, Inc. Lot: 3240674
- Spectra/Por® Biotech, regenerated cellulose dialysis membrane, MWCO: 15,000 Da. Spectrum Laboratories, Inc. Lot: 3226526
- Spectra/Por® Dialysis, dialysis membrane, MWCO: 50,000 Da. Spectrum Laboratories, Inc. Lot: 3227824
- Beakers, 300 mL
- Scissors
- Spectra/Por®, magnetic weighted closure. Reorder no.: 132760
- Spectra/Por®, closures. Reorder no.: 132736
- Terumo Schale®, plastic Petri dish, Terumo medical corporation

- Multi magnetic stirrer HSD-6, As One Corporation, Osaka, Japan, Lot: X81916

3.2.3.2 Centrifuge

- Centrifuge tube, 15 mL, Labcon, Lot: 6832-653C
- Hybrid Refrigerated Centrifuge 6200, Kubota Co., Tokyo, Japan

3.2.3.3 Filtration

- Dismic® Filter 0,2µm, Toyo Roshi Kaishi, Lt.d, Japan. Lot: 808071BD
- Dismic® Filter 0,45µm, Toyo Roshi Kaishi, Lt.d, Japan. Lot: 612212CD
- Dismic®-25cs Filter 0,45µm, Toyo Roshi Kaishi, Lt.d, Japan Lot: 801081CD
- Terumo® Syringe

3.2.3.4 Ultrafiltration

- Vivaspin 2, membrane: 5,000 Da, MWCO. Sartorius Stedim Biotech GmbH, Goettinghen, Germany. Lot: 08VS0248
- Vivaspin 2, membrane: 30,000 Da, MWCO. Sartorius Stedim Biotech GmbH, Goettinghen, Germany. Lot: 08VS0251
- Vivaspin 2, membrane: 100,000 Da, MWCO. Sartorius Stedim Biotech GmbH, Goettinghen, Germany. Lot: 07VS0227

3.2.4 *In vitro* drug release studies from FF-loaded micelles

- Spectra/Por® Biotech, regenerated cellulose ester dialysis membrane, MWCO: 5,000 Da. Spectrum Laboratories, Inc. Lot: 3232292

3.2.5 T_g, T_m and degradation of DP7, PEG_{5000}-PCL_{5000} and PEG_{4000} / DSC analysis

- Mettler Toledo, DSC822^e

3.2.6 Storage for samples

- Refrigator, Medicool, Sanyo, Tokyo, Japan
- Nihon freezer, Tokyo, Japan
- Airing cupboard, Sibata, Tokyo, Japan

3.2.7 Balance /scale

- Balance XS 205, Mettler Toledo, Ser.No. 1127110037
- Balance XS UMX2, Mettler Toledo, Ser. No. 1126093579

3.2.8 Pipettes used throughout the work

- Volume: 100-1000 µl, Nichipet EX Plus. NICHIRYO Co.,Ltd. Japan
  Lot:K08920601

- Volume: 1-10 ml, Nichipet EX Plus. NICHIRYO Co.,Ltd. Japan
  Lot: J07X26062

- Volume: 2-20 µl, Nichipet EX Plus. NICHIRYO Co.,Ltd. Japan
  Lot: K08918211

- Volume: 0.1-2 µl, Nichipet EX Plus. NICHIRYO Co.,Ltd. Japan
  Lot: J07X06842

- Volume: 20-200 µl, Nichipet EX Plus. NICHIRYO Co.,Ltd. Japan
  Lot: K08612901

3.3 Methods

3.3.1 Preparation of micelle solutions

3.3.1.1 Nanomizer method

In this method a specialized device, Nanomizer (NM) (Figure 10), is used to decrease particle size and hence form micelles. All materials e.g. 20 mg of DP7, 2 mg of SA, 2 mg of FF and 20 mL of ultra pure water were put in a glass jar. The solution was homogenized for about 15 minutes at 10.000 rpm. After homogenising, 20 mL of ultra pure water was added to the solution. Both filtration rate (FR) and temperature (T) were adjusted on the NM.

Two settings were used:

i. FR= 45 meters/sec and T = 25°C
ii. FR= 30 meters/sec and T = 25°C

Figure 10. The Nanomizer.

The solution was added directly into a metal-reservoir attached to the NM. The solution passed through the NM, 6 mL at a time, into a beaker. When the whole solution had been through the NM, it was placed in the metal-reservoir and passed through again. This process was repeated 10 to 200 times. To remove undesired bubbles, a long wire was pushed down the reservoir and through the intake of the NM. The NM was cleaned with 99,5 % ethanol after each session.

3.3.1.2 Co- solvent evaporation method

This method is based on drug and polymer dissolution in the same volatile water-miscible organic solvent (Rapoport, 2007). When the solvent is removed from the solution with evaporation, micelles are formed.
At first micelles containing only DP7 were prepared as follows:

20 mg of DP7 was put into a 100 mL recovery flask and dissolved in 1 mL of acetone. The recovery flask was fitted to a stand in a water bath at 51-54°C. Temperature was maintained by a magnetic stirrer. 40 mL of ultra pure water was added drop by drop into the stirring solution. The first 20mL of drops were added very slowly and the latter 20 mL a bit faster but with great care. This whole process took about 30 minutes. The remaining acetone was removed by evaporation at 45°C for 1 hour (Jette et al., 2004; Wilhelm et al., 1991). Pressure was scaled down slowly to prevent a suction- effect.
Micelles containing SA and FF were prepared as follows:
Stock solutions were prepared by dissolving 10 mg of SA or 10 mg of FF in 10 mL of acetone separately. The method was carried out as described above, with one exception; DP7 was dissolved in 2 mL of SA stock solution and 2 ml of FF stock solution instead of 1 mL as described above.

3.3.1.3 Thin film method

Stock solutions were prepared in the same manner as in the Co-solvent method (CSM). 20 mg of DP7 was dissolved in 2 mL of SA stock solution and 2mL of FF stock solution in a recovery flask. The solvent was removed by evaporation at 45°C for 1 hour to obtain a solid thin film (Wang, Yu, Han, Sha & Fang, 2007). After evaporation 40 mL of ultra pure water was added to the solution. The flask was vortexed for about 3 minutes or until the film had been removed from the surface of the glassware. Finally the solution was put in an ultrasonic bath for 10 minutes.

3.3.2 Characteristics of micelles

3.3.2.1 Quantification of stearic acid

This method is a sensitive, enzyme-based method for detecting the long chain of free fatty acids. Compared to conventional methods, this method has a high specificity for free fatty acids, is reproducible, is applicable to smaller quantities of sample, and can be completed within a shorter period of time. In the assay, SA and palmitic acid (PA) are converted to their CoA derivatives, which are subsequently oxidized with the

concomitant generation of colour (Mizuno, Toyosato, Yabumoto, Tanimizu & Hirakawa, 1980).

Free Fatty Acid Quantification Kit (BioVision) was used to quantify SA in micelle solutions. Instructions from the kit-manual were followed in most parts. Standard curves for SA as well as the preparation of samples were not performed by instructions from the kit.

Prior to preparation of standard curves reagents were prepared. Fatty Acid Probe was dissolved in 220 µL of dimethylsulfoxide (DMSO). Acyl-CoA Synthetase Reagent (ACSR) and Enzyme Mix (EM) were dissolved, individually in 220 µl of Assay Buffer (AB) each by pipetting up and down. For the standard curve of PA 0, 2, 4, 6, 8, 10 µL of PA was added into 96-well plate individually. Volume was adjusted to 50 µL per well with AB to generate 0, 2, 4, 6, 8, 10 nmol/well of the fatty acid standard.

Two methods were used to prepare the samples, one for liquid samples and one for dried samples. At first stock solutions of SA and DP7 were made. For SA stock-solution 10 mg of SA was dissolved in 10 ml of acetone (1 mg/ 1mL). As for DP7, 50 mg of DP7 was dissolved in 10 mL of acetone (5 mg/mL)

i. Sample preparation for liquid samples:

   Different volumes of liquid samples were added directly to each well in a 96-well plate and then the volume was brought up to 50 µL/well with Assay Buffer.

ii. Sample preparation for dried samples:

   Two samples were made, one with SA and DP7 and another one with SA only. This was done too see if DP7 interferes with the colorization of SA. In one microcentrifuge tube, 10 µl of SA stock solution was added and to another tube, 10 µl of SA stock solution and 20 µL of DP7 stock solution were added. The acetone was removed by drying the sample under argon. After drying, 200 µL of assay-buffer (AB) and 2 µL of Triton-X were used to dissolve the sample. The dried material was vortexed extensively for 3 minutes followed by an ultrasonic bath for 10 minutes. Different volumes, 40 µl (1.75 nmol), 20 µl (3.5 nmol) and 10 µl (7.0 nmol) of the sample was added to 4 wells and the volume brought up to 50 µL per well with AB. 2 µL of ACS was added to all standard and sample wells. The plate was shaken for 5 minutes and incubated at 37°C for 30 minutes.

While incubating, the Reaction Mix (RM) was prepared. For each well, 50 µL of RM was needed. Hence, the amount of RM made each time depended on the number of samples e.g. if 9 samples were to be tested 500 µL of RM was prepared. The plate was shaken for 5 minutes and incubated for 30 minutes at 37°C. Samples were protected from light by wrapping the plate in aluminium foil. Bubbles were removed by a heated glass pipette.
Fatty acids were quantified by colorimetric method. Spectrophotometry (λ) was set to 570 nm in a micro-plate reader.

3.3.2.2 Quantification of fenofibrate

FF was quantified by High-performance liquid chromatography (HPLC). The column used was Capell Pak C18, with N=14976. The mobile phase was a mixture of acetonitrile and ultra pure water (2:1(v/v)). The flow rate was 1mL/min and the injection volume was from 1 µL to 10 µL (injection volume of 10 µL was used in almost all cases). FF was detected by UV (288 nm) absorption. Total run time for each sample was 8 minutes.
Entrapment efficiency (EE) of FF was calculated from the ratio of drug amount in the micelles to the total drug amount added in the initial solution.

The drug EE of FF was calculated using the following equation:

$$EE=\left(\frac{\text{amount of FF in sample}}{\text{the total amount of FF}}\right) x100\%$$

3.3.2.3 Particle size

The particle size was measured by dynamic light scattering method using a Zetasizer Nano-ZS. Samples were diluted to the appropriate concentration with ultra pure water. For each sample, measurements were performed in triplicate to yield size and size distribution.

3.3.2.4 Gel filtration chromatography

Separation of particles of DP7 in micelle solution was performed by gel filtration chromatography using a Superose 6 HR 10/300 GL column together with a HPLC system. Flow rate was set at 0.5 mL/min. DP 7 was detected by UV (220 nm) absorption. Total run time for each sample was 60 minutes (Franzini et al., 2008).

3.3.2.5 CAC and CMC

The onset of micellization (CAC) of DP7 was measured by fluorescence spectroscopy in the presence of a hydrophibc porobe. Pyrene with a solubility of 0.8 µg/mL was used as a hydrophobic fluorescence probe (Shahla Jamzad & Reza Fassihi, 2006). At first a saturated pyrene-solution was prepared by diluting 1.18 mg of pyrene in 1 L of distilled water. The solution was stirred for 8 hours and protected from light. The solution was filtered through by 0.45 µm pore-size filter to remove excess undissolved pyrene microcrystals. Next, solution of DP7 was prepared by dissolving 6.30 mg of DP7 in 31.50 mL of saturated pyrene-solution. Stock solutions were diluted with pyrene-saturated water to obtain solutions of varying concentrations. Serial dilution is exemplified in Table 7.

Table 7. Serial dilution of DP7 and pyrene solution.

Sample	Concentration (mg/mL)	Volume of DP7 stock solution	Volume of saturated pyrene solution	Total volume
	2.0×10^{-1}			
1	1.0×10^{-1}	15 mL	15 mL	30 mL
2	8.0×10^{-2}	4 mL	1 mL	5 mL
3	6.0×10^{-2}	3 mL	2 mL	5 mL
4	4.0×10^{-2}	2 mL	3 mL	5 mL
5	3.0×10^{-2}	1.5 mL	3.5 mL	5 mL
6	2.0×10^{-2}	1 mL	4 mL	5 mL
7	1.5×10^{-2}	0.75 mL	4.25 mL	5 mL
8	1.0×10^{-2}	3 mL	27 mL	30 mL
9	8.0×10^{-3}	4 mL	1 mL	5 mL
10	6.0×10^{-3}	3 mL	2 mL	5 mL
11	4.0×10^{-3}	2 mL	3 mL	5 mL
12	2.0×10^{-3}	1 mL	4 mL	5 mL
13	1.0×10^{-3}	3 mL	27 mL	30 mL
14	8.0×10^{-4}	4 mL	1 mL	5 mL
15	6.0×10^{-4}	3 mL	2 mL	5 mL
16	4.0×10^{-4}	2 mL	3 mL	5 mL
17	2.0×10^{-4}	1 mL	4 mL	5 mL
18	1.0×10^{-4}	3 mL	27 mL	30 mL
19	4.0×10^{-5}	2 mL	3 mL	5 mL
20	1.0×10^{-5}	0.5 mL	4.5 mL	5 mL

Excitation spectra were monitored at λ_{em} = 390 nm at 25°C with fluorescence spectrophotometer (Francis, Piredda & Winnik, 2003).

CMC:

The onset of micellization or CMC of DP7 was measured by determination of surface tension, in the absence of a hydrophobic probe. DP7 stock solution (0.7 mM) was prepared by diluting 43.84 mg of DP7 in 10 mL of ultra pure water. From this stock solution, 0.68 µM – 0.35 mM of DP7 sample-solutions were prepared by serial dilutions (twice diluted in each step).

100 µL of DP7 solutions were put into a 96-well plate and surface tension was determined with Delta-8.

3.3.3 Separation of ideal micelles from other particles in micelle solution

Initially, a dialysis method was used to isolate "ideal" micelle particles from other particles. For this purpose, semi-permeable membranes with variable pore-size rating, referred to as Molecular Weight Cut Off (MWCO) were used. This experiment failed.

Secondly, a method with Vivaspin 2 was used. This method was performed in three steps or the threestep method shown in Figure 11.

Figure 11. The threestep method. The method is used to isolate "ideal" micelle particles from other particle s in a micelle solution.

3.3.3.1 Centrifugation

Samples were placed in a centrifuge tube and centrifuged at 3000 rpm for 10 minutes.

3.3.3.2 Filtration

After centrifugation, samples were filtered through 0.2 µm pore-sized filter.

3.3.3.3 Ultrafiltration

Vivaspin 2 (Figure 12) was used to ultrafiltrate the samples. Vivaspin is an utrafiltration device that can handle samples up to 2 mL. Vivaspin 2, with MWCO 100.000 Da was selected as the most appropriate membrane. The concentrator was filled up to maximum volume (approx. 1.8 mL) with a filtered and centrifuged solution. The concentrator was centrifuged at 3500 rpm for 10 minutes. After centrifugation, the concentrate was spun in reverse into the concentrate recovery cap, at 3000 rpm for 2 minutes.

Figure 12. Vivaspin 2. Obtained from the website "www.sartorius-stedim.com/index.php?id=2570" on April 22, 2009.

3.3.4 *In vitro* drug release studies from FF-loaded micelles

In vitro FF release from micelles was investigated using phosphate buffer solution with different pH (1.2, 6.8, 7.4) as dissolution medium (Ye et al., 2008). 3 mL of freshly prepared FF-loaded micelles were placed in a Spectro/Por dialysis bag with MWCO = 5000 Da, which was then put into a 300 mL buffer solution with different pH in a beaker. The beakers were incubated at 37°C and stirred. Samples of 50 µL were taken from the dialysis bag. FF, in each sample, was quantified as described above and the percentage of released drug was calculated (Velluto et al., 2008).

3.3.5 T_g, T_m and degradation of DP7, PEG_{5000}-PCL_{5000} and PEG_{4000} / DSC analysis

T_g, T_m and degradation of DP7, PEG_{5000}-PCL_{5000} and PEG_{4000} was determined by differential scanning calorimetry (DSC) method. Samples were prepared by weighing approximately 5 to 6 mg of DP7, PEG_{5000}-PCL_{5000} or PEG_{4000} into aluminium pans which were sealed with aluminium caps (Klose et al., 2009). DSC was used with the following method: heating rate was 10° C min^{-1}, from -100 °C to 220°C beneath nitrogen-gas.

4. RESULTS

4.1 Characteristics of DP7

4.1.1 Preliminary studies of preparation of DP7 micelles

Throughout the work on DP7-micelle solutions, a number of formulations and/or composition of surfactant (DP7 and poly(ethylene oxide-β-caprolactone) stearic acid and a hydrophobic drug (fenofibrate) were made to evaluate which ratio was suitable to yield micelles.

Furthermore, two settings (filtration rate and temperature) on the NM were tested to find out which settings were suitable for the preparation of di-block copolymer micelles. Since there are no published data about using the nanomizer method (NMM) in micelle preparation it was also necessary to observe how many repetitions were needed to make the solutions transparent and form micelles.

The evaluation was dependent on particle size and transparency. For particle size, data was obtained from both non-filtered and filtered samples and all measurements were performed in triplicate to yield size and size distribution.

Table 8 to Table 12 show different compositions of materials and settings on the NM.

Table 8. Particle size. Average particle size and polydispersity index (PdI) of DP7 micelle solutions, prepared with the Nanomizer method, containing 100 mg of DP7 and 20 mL of ultra pure water.

		Particle size (nm)	
Sample nr.	**Repetition through NM**	**Not filtered**	**Filtered (20µm filter)**
1	**0**	**280 ± 0.975**	
2	**10**	**432 ± 0.812**	
3	**20**	**288 ± 0.542**	**42.6 ± 0.460**
4	**30**	**415 ± 0.458**	
5	**40**	**621 ± 0.405**	**35.7 ± 0.383**
6	**50**	**776 ± 0.565**	**31.8 ± 0.368**

[a]Settings on the Nanomizer (NM): Filtration rate = 25 meters/ second, temperature = 5°C

Table 9. Particle size. Average particle size and polydispersity index (PdI) of DP7 micelle solutions, prepared with the Nanomizer method, containing 1 mg of stearic acid, 100 mg of DP7 and 20 mL of ultra pure water.

		Particle size (nm)	
Sample nr.	**Repetition through NM**	**Not filtered**	**Filtered (20µm filter)**
1	**0**	**206 ± 0.880**	**72 ± 0.552**
3	**30**	**320 ± 0.415**	**88.5 ± 0.385**
4	**60**	**266 ± 0.424**	**45.1 ± 0.505**
5	**100**	**482 ± 0.486**	**60.8 ± 0.566**
6	**130**	**335 ± 0.425**	**43.3 ± 0.516**

[a]Settings on the Nanomizer (NM): Filtration rate = 25 meters/second, temperature = 5°C

Table 10. Particle size. Average particle size and polydispersity index (PdI) of DP7 micelle solutions, prepared with Nanomizer method (NMM), containing 150 mg DP7, 1.5 mg of stearic acid and 30 mL of ultra pure water.

		Particle size (nm)	
Sample nr.	**Repetition through NM**	**Not filtered**	**Filtered (20µm filter)**
1	**30**	**633 ± 0.091**	**35.5 ± 0.374**
2	**50**	**496 ± 0.651**	**34.9 ± 0.383**
3	**70**	**416 ± 0.459**	**44.2 ± 0.486**
4	**100**	**446 ± 0.825**	**40.3 ± 0.486**

[a]Settings on the Nanomizer (NM): Filtration rate = 45 meters/second, temperature = 25°

Table 11. Particle size. Average particle size and polydispersity index (PdI) of DP7 micelle solutions, prepared with Nanomizer method (NM), containing, 100 mg of DP7, 4 mg of stearic acid and 20 mL of ultra pure water.

		Particle size (nm)	
Sample nr.	**Repetition through NM**	**Not filtered**	**Filtered (20µm filter)**
1	**0**	**339 ± 0.452**	**70.3 ± 0.535**
2	**10**	**277 ± 0.484**	**53.7 ± 0.467**
3	**30**	**390 ± 0.520**	**76.9 ± 0.548**
4	**50**	**361 ± 0.506**	**62.4 ± 0.552**
5	**60**	**328 ± 0.645**	**85 ± 0.592**
6	**100**	**426 ± 0.498**	**71.1 ± 0.569**
7	**200**	**397 ± 0.469**	**70 ± 0.578**

[a]Settings on Nanomizer (NM): Filtration rate = 25 meters/second, temperature = 5°C

Table 12. Particle size. Average particle size and polydispersity index (PdI) of micelle solutions, prepared with Nanomizer method (NMM), containing 20 mg of DP7, 1mg of FF and 40 mL of ultra pure water.

		Particle size (nm)	
Sample nr.	**Repetition through NM**	**Not filtered**	**Filtered (20µm filter)**
1	**10**	**717 ± 1.000**	**75.4 ± 0.522**
2	**30**	**569 ± 0.608**	**44.1 ± 0.425**
3	**50**	**473 ± 0.479**	**81 ± 0.513**
4	**80**	**442 ± 0.533**	**52.2 ± 0.446**

[a]Settings on Nanomizer (NM): Filtration rate = 30 meters/second, temperature = 25°C

4.1.2 Comparison of DP7 and poly(ethylene oxide-β-caprolactone)

DP7 micelles and PEG-PCL micelles with different PEG/PCL ratios were compared regarding particle size and transparency. For each sample, data was obtained from non-filtered and filtered samples and all measurements were performed in triplicate to yield particle size and distribution. Table 13 summarizes the average micelle size of the micelle solutions. Each solution contained 20 mg of surfactant and 20 mL of ultra pure water. The DP7 micelles were the smallest (Figure 13 and Table 13) of the micelles and the PEG_{5000}-PCL_{5000} yielded the second smallest particle size. For all five polymer concentrations examined, the micelle size varied from 31.8 nm to 496 nm, for the filtered samples.

Table 13. Mean particle size of micelles formed by the Nanomizer method after various repetitions through the NM. All samples were filtered through 0.2 µm pore-sized filter.

Sample	Particle size mean average ± PdI (nm)[a]			Transparency Yes or No
	20 RP[b]	40 RP[b]	50 RP[b]	
PEG_{5000}-β-PCL_{2300}	154 ± 0.249	122 ± 0.099	122 ± 0.087	Yes
PEG_{5000}-β-PCL_{5000}	137 ± 0.246	97.2 ± 0.084	97.4 ± 0.113	Yes
PEG_{5000}-β-PCL_{11000}	123 ± 0.180	113 ± 0.128	112 ± 0.130	No
PEG_{5000}-β-PCL_{18000}	496 ± 0.436	186 ± 0.277	225 ± 0.407	No
DP7	42.6 ± 0.460	35.7 ± 0.383	31.8 ± 0.368	Yes

[a] Size of micelles determined by dynamic light scattering measurement
[b] Number of repetitions through the Nanomizer

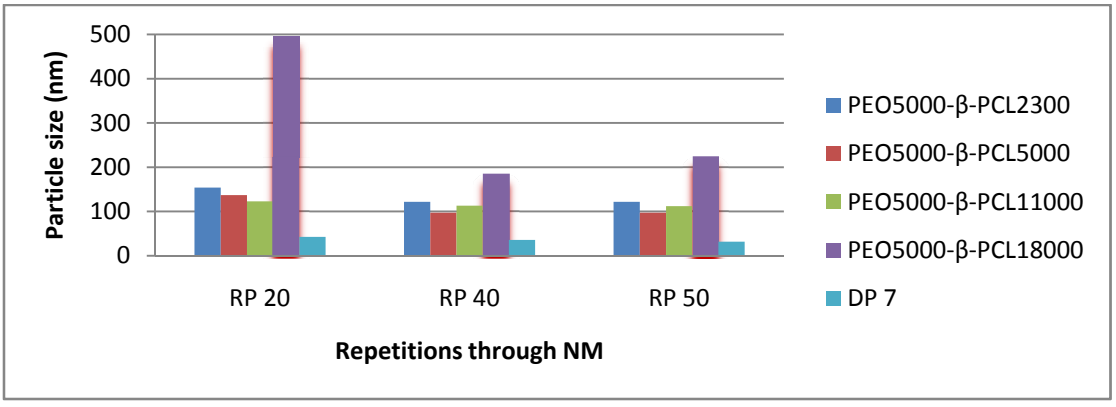


Figure 13. Average particle size yielded from various block copolymers. The polydispersity indexes (PdI) are present on the graph but are too small to be noticed.

Since PEG_{5000}-PCL_{5000} yielded the smallest particle size of the PEG/PCL it was chosen as comparative material to DP7.

4.1.3 T_g, T_m and degradation of DP7, PEG_{5000}-PCL_{5000} and PEG_{4000}

Thermal analysis was carried out by DSC. T_g and T_m were determined for three block polymers, DP7, PEG_{5000}-PCL_{5000} and PEG_{4000}. At the glass transition temperature (T_g), a small endothermal reaction was observed as the specific heat of the copolymer increased. On further heating, another endothermal reaction, much larger than the first one, was observed as the polymer melted. The last peak was observed where degradation occurred. The degradation graph had broad peaks because DP7, PEG_{5000}-PCL_{5000} and PEG_{4000} are polymers end hence do not have a single degradation temperature. The results from DSC analysis are listed in Table 14 through 16.
A preview of a measurement data sheet is shown in Appendix 1.

Table 14. Glass transition (T_g) temperature of DP7, PEG_{5000}-PCL_{5000} and PEG_{4000}.

Polymer	T_g[a] (°C)	T_g[a] (°C)	T_g[a] (°C)	Mean T_g (°C)	SD[b]
DP7	-31.77	-31.25	-32.03	-31.51	± 0.37
PEG_{5000}-PCL_{5000}	-33.34	-34.65	-35.44	-34.48	± 1.06
PEG_{4000}	-39.64	-37.81	-38.59	-38.68	± 0.92

[a]Measured by DSC analysis
[b]Standard deviation

Table 15. Melting point (T_m) of DP7, PEG_{5000}-PCL_{5000} and PEG_{4000}.

Polymer	T_m[a] (°C)	T_m[a] (°C)	T_m[a] (°C)	Mean Tm (°C)	SD[b]
DP7	54.09	53.3	52.63	53.70	± 0.56
PEG_{5000}-PCL_{5000}	61.02	61.13	60.85	61.00	± 0.14
PEG_{4000}	60.65	60.14	60.17	60.32	± 0.29

[a]Measured by DSC analysis
[b]Standard deviation

Table 16. Degradation (DG) of DP7, PEG_{5000}-PCL_{5000} and PEG_{4000}.

Polymer	DG[a] (°C)	DG[a] (°C)	DG[a] (°C)	Mean DG (°C)	SD[b]
DP7	154.81	156.14	155.81	155.48	± 0.94
PEG_{5000}-PCL_{5000}	159.5	156.33	155.17	157.00	± 2.24
PEG_{4000}	143.67	141.16	141.16	142.00	± 1.45

[a]Measured by DSC analysis
[b]Standard deviation

4.1.4 CAC and CMC of DP7 micelles

4.1.4.1 CAC

The CAC was determined by fluorescence assay, based on changes in fluorescence intensity ratio $I_{335.4}/I_{133.4}$ of pyrene, a hydrophobic probe added in minute amounts to a polymer solution. The pyrene method was chosen due to its functional, versatile and easy application. A plot of average $I_{335.4}/I_{133.4}$ (I_3/I_1) ratios with standard deviations versus the logarithm of DP7 concentration is shown in Figure 14.

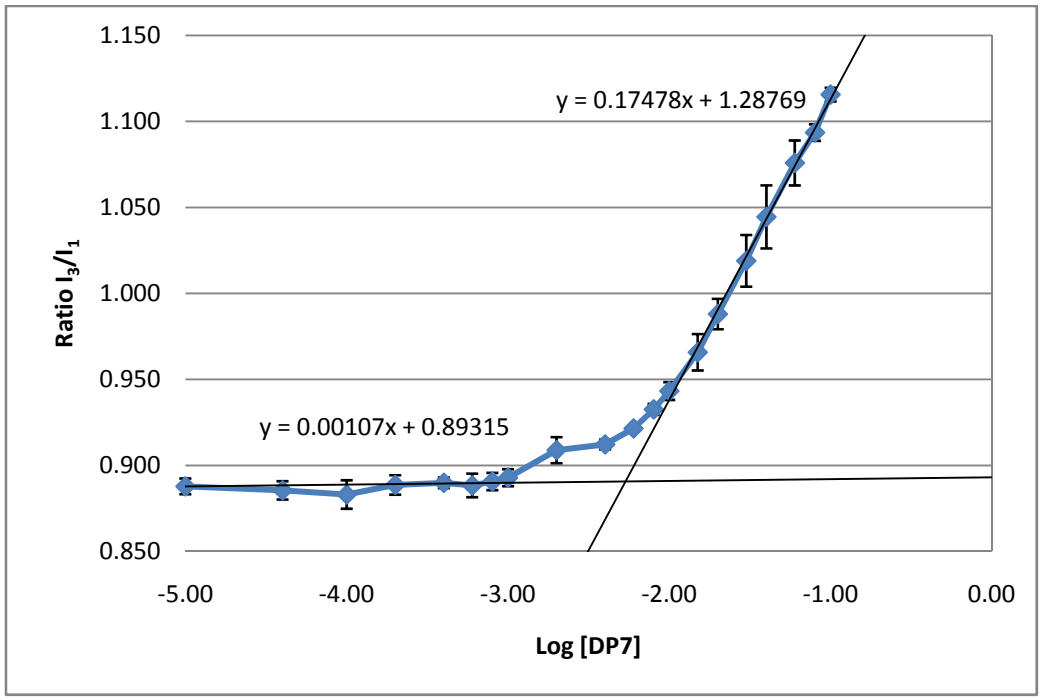


Figure 14. The critical association concentration (CAC). Changes in the $I_{335.4}/I_{133.4}$ ratio of pyrene fluorescence intensity as a function of concentration.

The CAC value was determined by two straight lines, one horizontal with an almost constant value of ratio $I_{335.4}/I_{133.4}$ and the other line approximating the steep upward section of the curve. The point of intersection was calculated according to equations shown in Figure 14.

The CAC value for DP7 was 5.65 x 10^{-3} mg/mL which equals 9.09 x 10^{-7} M.

4.1.4.2 CMC

The CMC was determined by surface tension measurments, in the absence of a hydrophobic probe.

A plot of average values from three measurements with standard deviations versus the logarithm of DP7 concentration is shown in Figure 15.

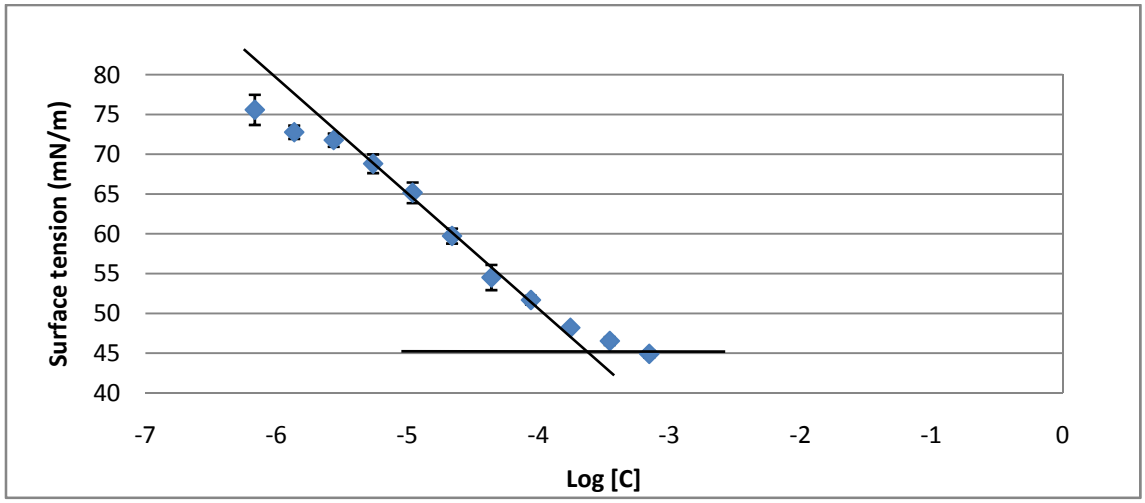


Figure 15. The critical micelle concentration (CMC) of DP7.

The CAC value was determined by two straight lines. The CMC value was estimated where the lines cross the x-axis.

The CMC value of DP7 was 0.245 mM which equals 1.52 mg/mL.

4.1.5 Comparison of methods for the preparation of DP7 micelles

To compare the potential for the formation of a transparent, aqueous DP7 micelle solution, three different methods, i.e., the NMM, the thin film method (TFM) and the CSM, were used to prepare micelle solutions (Table 17 and Figure 16). To evaluate which method is the most suitable, micelles prepared with each method were compared with two criterias:

i. transparency
ii. particle size

Table 17. Mean particle size yielded from micelle solution of 20 mg of DP7 and 1mg fenofibrate made with three methods; The Nanomizer method, the Thin film method and the Co–Solvent evaporation method.

Method	Average particle size ± PdI (nm)[a]		Transparency
	Not filtered	Filtered 0.20 µm	Yes or No
Nanomizer	442 ± 0.533	51.2 ± 0.195	Yes
Thin film method	132 ± 1.00	35.4 ± 0.291	Yes
Co–solvent evaporation method	197 ± 0.641	28.5 ± 0.254	Yes

[a] The average particle size of micelles was determined by dynamic light scattering measurement

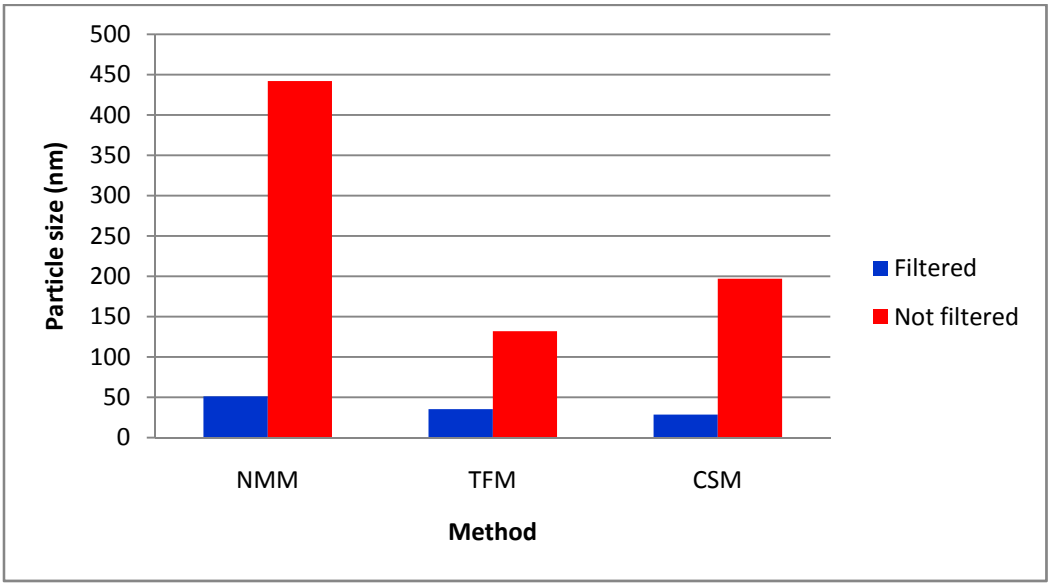


Figure 16. Average particle size yielded from three different methods, Nanomizer method (NMM), thin film method (TFM) and the co-solvent method (CSM).

4.2 The effect of stearic acid on micelle efficacy

To evaluate the effect of SA on micelle efficacy, micelle solutions containing SA were compared to micelle solutions without SA. The micelle solutions were compared in three aspects to evaluate the effect of SA:

i. Particle size
ii. Drug entrapment
iii. Increase of amount of FF in the micelle solution

4.2.1 Particle size

The effect of SA on particle size is shown in Figure 17 and Figure 18. Figure 17 also shows the mean particle sizes of micelle solutions containing only DP7.

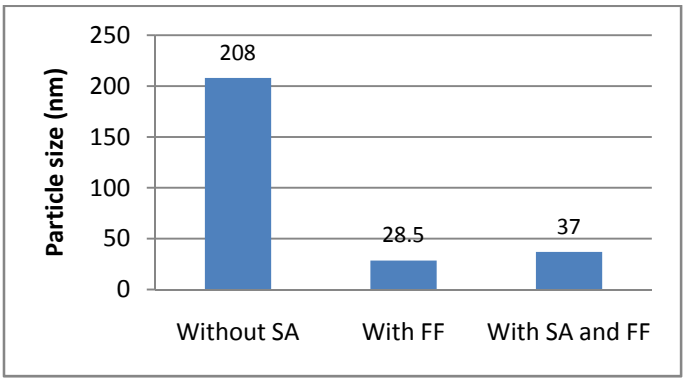


Figure 17. The effect of stearic acid (SA) on particle size. The first solution contains 20 mg of DP7, the second solution contains 20 mg of DP7 and 1 mg of fenofibrate (FF) and the third solution contains 20 mg of DP7, 1 mg of SA and 1 mg of FF. All the solutions contained 40 mL of ultra pure water and were prepared by the co-solvent method.

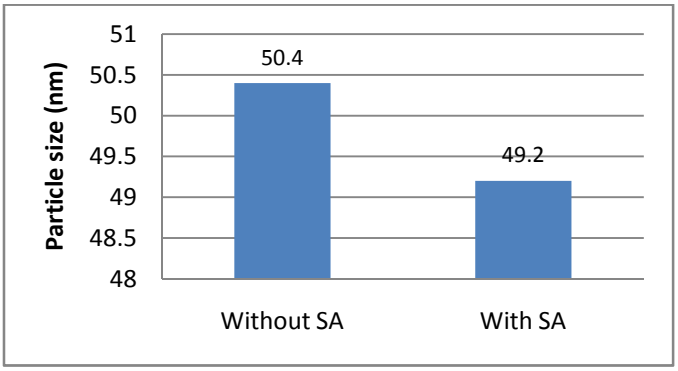


Figure 18. The effect of stearic acid (SA) on particle size. First solution contains 20 mg of DP7 and 1.5 mg of fenofibrate (FF) and the second solution contains 20 mg of DP7, 2 mg of SA and 2 mg of FF. All the solutions contained 40 mL of ultra pure water and were prepared by the thin film method.

4.2.2 Drug entrapment

The effect of SA on drug entrapment was evaluated in both DP7 micelles as in PEG_{5000}-PCL_{5000} micelles containing FF and SA (Table 18). The micelle solutions were prepared by the NMM.

Table 18. Manufacturing formula.

	Manufacturing formula:
I.	**1 mg of FF**
II.	**1 mg of SA**
III.	**20 mg of DP7**
IV.	**40 mL of ultra pure water**

Drug entrapment of FF was much higher in PEG_{5000}-PCL_{5000} micelles without SA both in filtered and un-filtered samples (Table 19 and Table 20). On the other hand, SA increased the amount entrapped by the DP7 micelles in samples that were not filtered, but made no difference in the filtered samples (Table 19 and Table 20).

Table 19. Drug entrapment of DP7 vs. PEG-PCL without stearic acid.

Without stearic acid			
DP7		PEG-PCL	
filtered	not filtered	filtered	not filtered
9.48 %	70%	29.9 %	91.8 %

Table 20. Drug entrapment of DP7 vs. PEG-PCL with stearic acid.

With stearic acid			
DP7		*PEG-PCL*	
filtered	not filtered	filtered	not filtered
7.76 %	94%	19.6 %	62.6 %

Drug entrapment efficiency using three different methods:

The effect of SA on micelle drug entrapment by three different methods was evaluated. For the NMM, SA had a negative effect as seen before. The drug entrapment by the CSM was slightly increased in the sample that was not filtered and the opposite effect was seen in the filtered sample (Table 21, Table 22 and Figure 19)

In the TFM, however, there was a tremendous increase in drug entrapment in the filtered sample that contained SA. The drug entrapment was increased by 473% in the filtered sample with SA compared to the sample that lacked SA (Table 21, Table 22 and Figure 19).

Table 21. The drug entrapment of DP7 with three different methods: the thin film method, the co-solvent method and the nanomizer method.

Without stearic acid					
Thin film method		Co-solvent evaporation method		Nanomizer method	
Filtered	Not filtered	Filtered	Not filtered	Filtered	Not filtered
9.56 %	80.1 %	9.56 %	80.1 %	29.9 %	91.8 %

Table 22. The drug entrapment of DP7 with three different methods: the thin film method, the co-solvent method and the nanomizer method.

With stearic acid					
Thin film method		Co-solvent evaporation method		Nanomizer method	
Filtered	Not filtered	Filtered	Not filtered	Filtered	Not filtered
54.77 %	95.7 %	9.52 %	85.9 %	7.74 %	94.2 %

The effect of cholesterol was also tested. The solutions containing cholesterol became cloudy and the particle size large. Hence, the solution was not tested further.

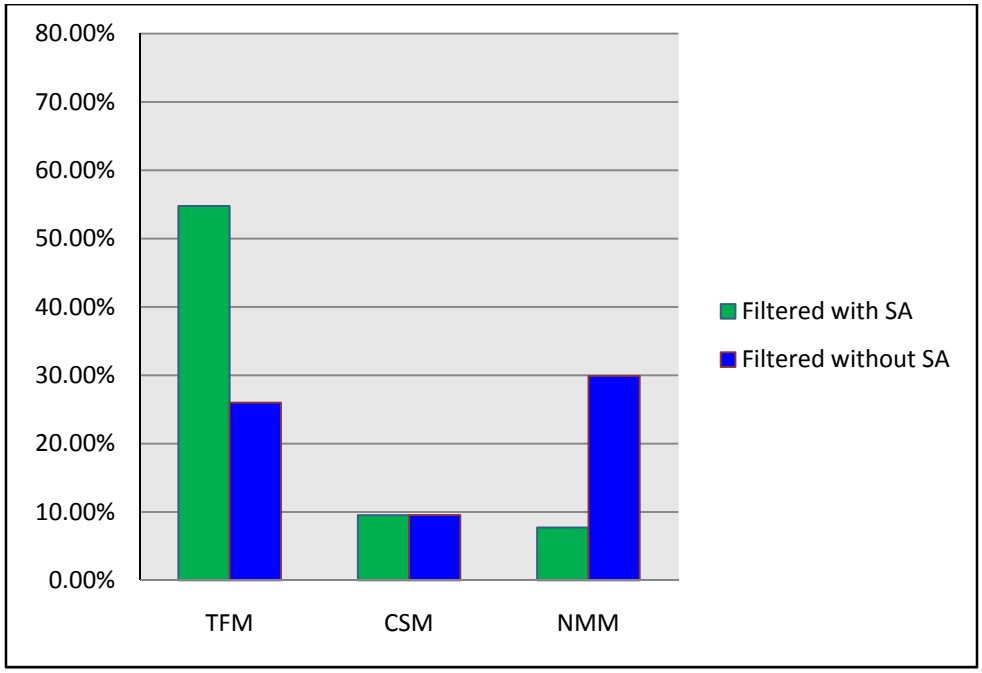


Figure 19. Drug entrapment efficiency of DP7 micelles with three different methods: the Nanomizer method (NMM), the thin film method (TFM) and the co-solvent method (CSM).

4.2.3 Increase of fenofibrate in micelle solutions

The purpose of this experiment was to survey if it was possible to enhance the amount of FF in a micelle solution in both DP7 and PEG-PCL micelles and hence, increase the amount of drug incorporated in the micelles. A new formulation was tried out (Table 23), containing double the amount of FF compared to what had been tried before.

Table 23. Manufacturing formulas of micelle solutions of DP7 and PEG_{5000}-PCL_{5000} with increased amount of FF.

Manufacturing formulas			
I.	**2 mg of FF**	**I.**	**2 mg of FF**
II.	**1 mg of SA**	**II.**	**1 mg of SA**
III.	**20 mg of DP7**	**III.**	**20 mg of PEG_{5000}-PCL_{5000}**
IV.	**40 mL of ultra pure water**	**IV.**	**40 mL of ultra pure water**

Outcome of PEG_{5000}-PCL_{5000} and DP7 micelles containing double amount of FF:
This test was performed by two methods, the TFM and the CSM.
The experiment failed. The solutions were cloudy and big particles adhered on the glassware.

Another manufacturing formula (Table 24) was tried, this time the amount of SA was also doubled:

Table 24. Manufacturing formulas of micelle solutions of DP7 and PEG_{5000}-PCL_{5000}.

Manufacturing formulas			
I.	**2mg of FF**	**I.**	**2mg of FF**
II.	**2 mg of SA**	**II.**	**2 mg of SA**
III.	**20 mg of DP7**	**III.**	**20 mg of PEG_{5000}-PCL_{5000}**
IV.	**40 mL of ultra pure water**	**IV.**	**40 mL of ultra pure water**

Outcome for PEG_{5000}-PCL_{5000} containing twofold amount of FF and SA:
The solution containing PEG_{5000}-PCL_{5000} became cloudy and contained big particles as before.

Outcome for DP7 containing twofold amount of FF and SA:
The solution became transparent. Hence, continued research was performed on micelle solutions performed by the TFM containing 2 mg of FF, 2mg of SA, 20 mg of DP7 and 40 mL of ultra pure water.

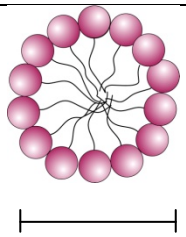
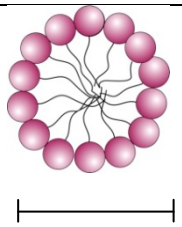
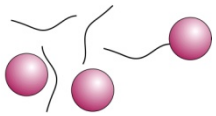
4.2.3.1 Quantification of SA

To quantify SA in a micelle solution a kit from BioVision was used. By following the instruction from the kit a standard curve was done for PA. Additionally, a standard curve for SA with and without DP7 was calculated. Several experiments were performed but all of them failed.

4.3 Isolation of "ideal" micelles from other particles in a micelle solution and characterization of DP7 micelles prepared with TFM

A micelle solution of DP7 containing FF was expected to contain 4 types of particles as shown in Table 25. Only one of the 4 types is an "ideal" particle and therefore a separation of the ideal one from the others was necessary. Samples of "un-ideal" and "ideal" micelle solutions are shown in Appendix 2.

Table 25. Types of particles in a micelle solution and methods to discharge the "un-ideal" particles.

Powder particles	Large micelles	Small micelles	Monomers
	 >100nm	 < 100nm	
Discharged by Centrifuge	Discharged by Filtration	"Ideal particle"	Discharged by Vivaspin 2

First, a dialysis method was used to isolate "ideal" micelle particles from other particles. The dialysis test failed.

Secondly, a method with Vivaspin 2 was used. This method was performed with the threestep method, described in method chapter no. 3.3.3.

Three measurements were performed in order to evaluate if the remaining solution contained micelles:

i. particle size and distribution
ii. quantification of FF
iii. particle distribution of DP7 by gel filtration chromatography

4.3.1 Particle size and distribution

The particle size of "ideal" micelles was measures as before in triplicate to yield size and size distribution in both volume and in intensity (Figure 20 and Figure 21).
The average particle size in an "ideal micelle solution was 24.5 nm (± 0.266).

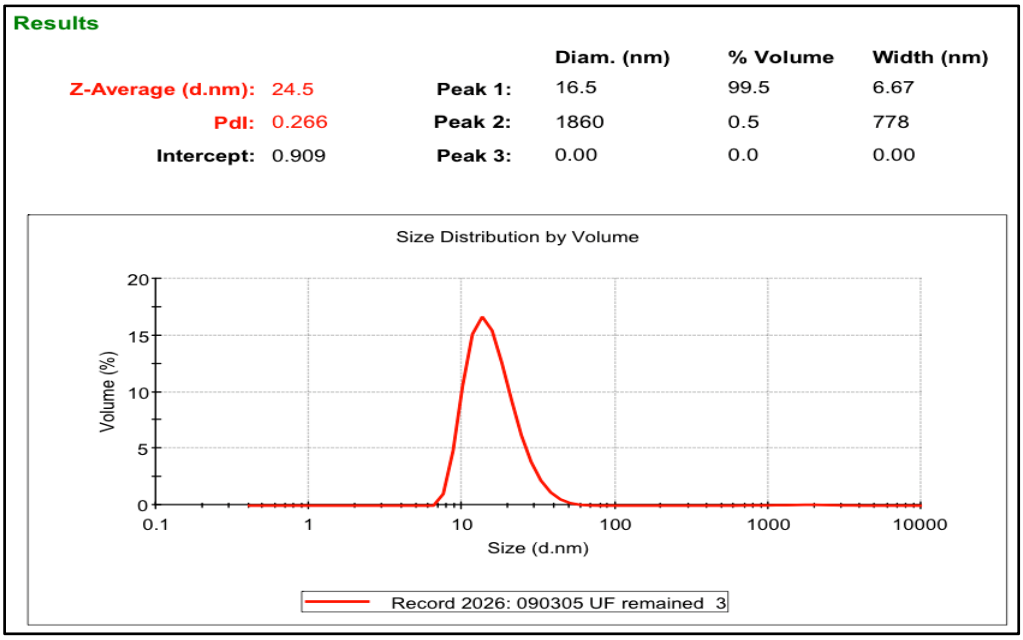


Figure 20. Average size of "ideal" micelles and size distribution by volume.

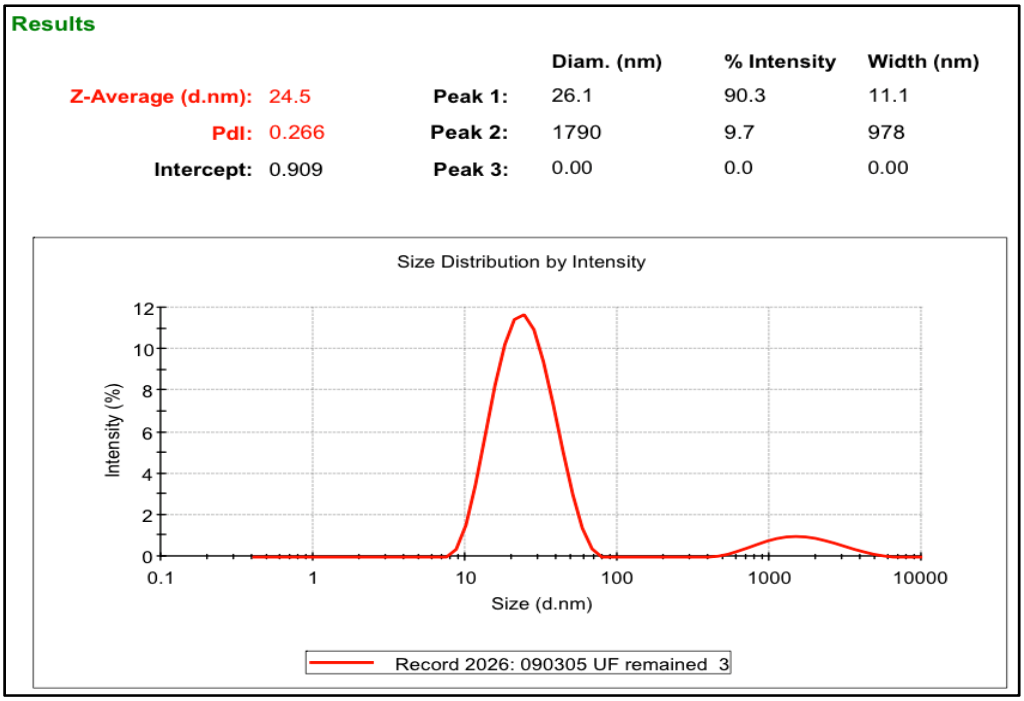


Figure 21. Average size of "ideal" micelles and size distribution by intensity.

Figure 17 shows size distribution by volume. Only one peak appears, containing 99.5% of all the particles, which is exemplary. For particle size distribution be intensity, two peaks appear, one large and contains 90.3% of all the particles and the other one, a very small peak, containing only 9.7 % (Table 20).

4.3.2 Quantification of FF

Quantification of FF was determined by HPLC systems. Figure 22 to Figure 24 show the amount of FF before and after ultrafiltartion with Vivaspin 2. Figure 22 shows the amount of FF before the ultrafiltration but after centrifugation and filtration through 0.2 µm pore-size filter. Figure 23 shows the amount of FF in the remained "ideal" micelle solution. The peaks with retention times around 6.8 minutes show FF (the peak with retention time at approx. 1.8 is acetone). Figure 24 shows the amount of FF in the solution that passed the membrane of the Vivaspin 2 (containing only monomers).

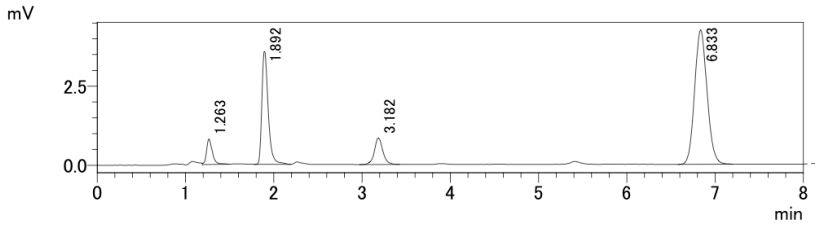


Figure 22. Amount of fenofibrate in a solution that has been centrifuged and filtrated.

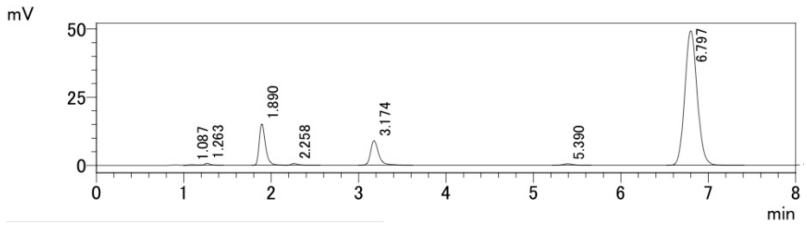


Figure 23. Amount of fenofibrate in an "ideal" micelles solution after ultrafiltration.

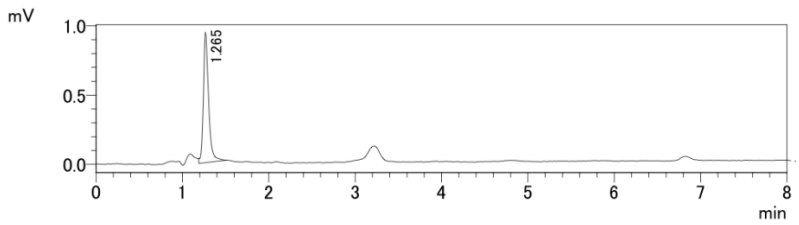


Figure 24. Amount of fenofibrate in a "passed" solution that contains only monomers. No peak appears at retention time 6.8 minutes, hence there was no fenofibrate in the solution.

4.3.3 Particle distribution of DP7 by gel filtration chromatography

For exactly the same test solutions, as were FF was quantified in and are shown in Figure 22Figure 24, DP7 particle distribution was determined by gel filtration chromatography. Figure 25 to Figure 27 show particle distribution of DP7 before and after ultrafiltration with Vivaspin 2. The peaks with retention times approx. 23 minutes are DP7.

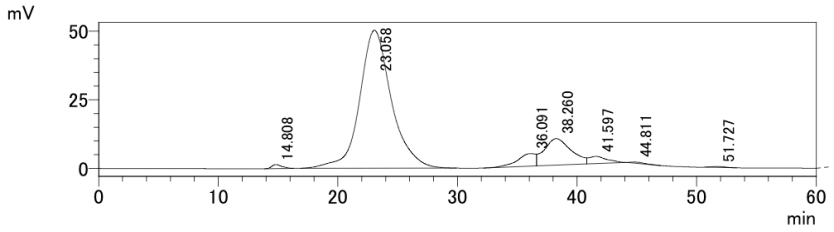


Figure 25. Particle distribution of DP7 in a solution that has been centrifuged and filtrated. The large peak at retention time at approx. 23 minutes was DP7.

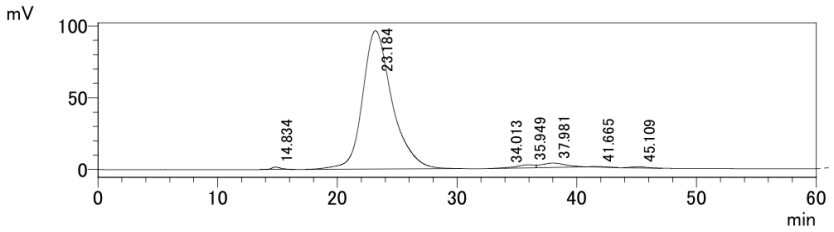


Figure 26. Particle distribution of DP7 in an "ideal" micelles solution after ultrafiltration. The large peak at retention time at approx. 23 minutes was DP7.

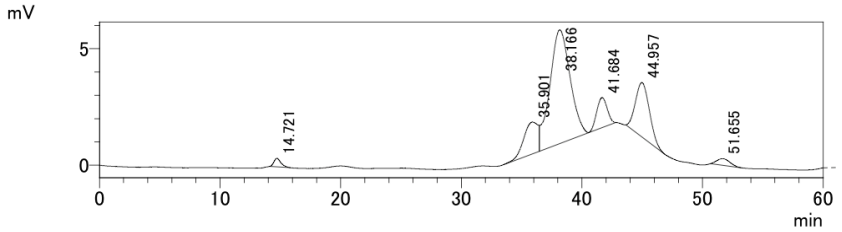


Figure 27. Particle distribution of DP7 in a "passed" solution that contains only monomers. No peak appears at approx. 23 minutes, hence there is no DP7 in the solution.

4.4 *In vitro* drug release test from fenofibrate loaded DP7 micelles

Dialysis is a reliable way to estimate the release of drugs from micelles. At first a release test was performed with distilled water as a reservoir.
This test failed. Hence, another test was carried out.

Secondly, buffers were used as reservoirs. Keeping in mind the physiological condition of the human body, drug release tests of FF loaded DP7 micelles were performed at various pH (1.2, 6.8, and 7.4), to mimic *in vivo* conditions. The results are presented in Figure 28, Figure 29 and Figure 30.

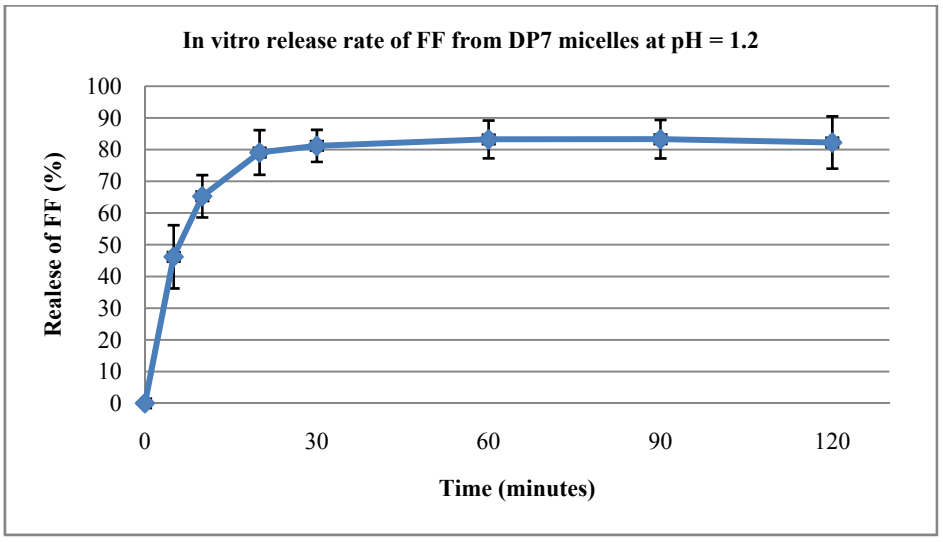


Figure 28. *In vitro* drug release from DP7 micelles at pH = 1.2.

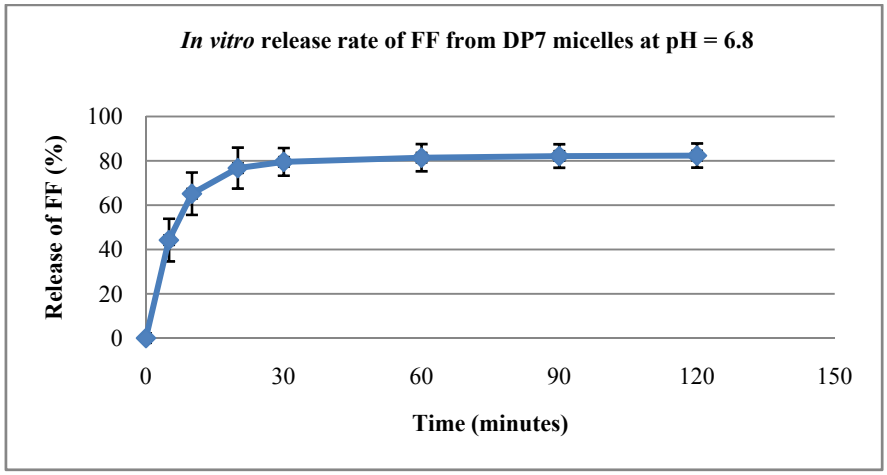


Figure 29. *In vitro* drug release from DP7 micelles at pH = 6.8.

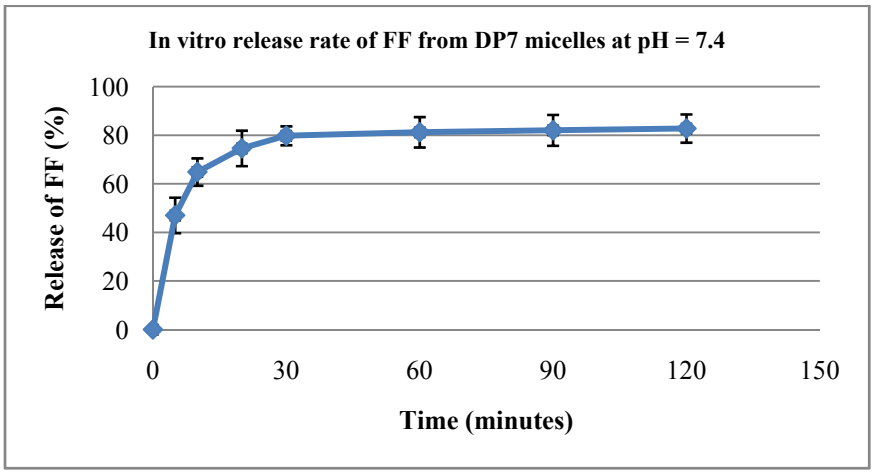


Figure 30. *In vitro* drug release from DP7 micelles at pH = 7.4.

The drug release rate turned out to be very similar at all three pH values. After 120 minutes the micelles at pH 1.2 had released 82.24 % of their cargo, micelles at pH 6.8 had released 82.39 % of their cargo and micelles at pH 7.4 had released 82.76% of their cargo.

4.5 Enhanced solubility of FF by DP7 micelles

FF was quantified in a pure "ideal" micelle solution by HPLC.

Amount of FF in 1 mL solution was:
150.16 µg/mL

Enhanced solubility compared to FF in water is (calculated from the lowest and highest water solubility of FF found in references):
((150.16 – 0.1) µg/mL)/ (0.1 µg/mL) = 1500.6
((150.16 -0.8) µg/ mL) / 0.8 µg/mL) = 186.7

The enhanced solubility of FF was 190 to 1500 fold compared to its water solubility.

5. DISCUSSION

Micelles formed from amphiphilic block copolymers have been receiving much attention as nanocarriers in the field of drug delivery. Micellar solubilization is a powerful option for dissolving poorly soluble drugs within a hydrophobic micellar core surrounded by a hydrophilic shell. DP7 is a novel di-block copolymer that self-aggregates to form nano-size micelles and is a promising carrier for drugs with challenging properties. There is no published data on DP7; hence the results in this project were compared to commercial block copolymers.

5.1 Characteristics of DP7

5.1.1 Preliminary studies of preparation of DP7 micelles

The preliminary studies of DP7 implied that DP7 is a good alternative for micellization as it yielded a minute particle size and was easy to handle. The nanomizer method (NMM) was promising, and it has a considerable advantage over all the other micelle preparation methods, i.e. there is no need for any solvents. The NMM is also very easy in application. The disadvantage of the method is a rather time-consuming procedure.

5.1.2 Comparison of DP7 and Poly(ethylene Oxide-β-caprolactone)

The DP7 micelles were the smallest of all micelles compared in the research. Since there was limited time, only one poly(ethylene oxide-β-caprolactone) was chosen for further comparison.

The PEG_{5000}-PCL_{5000} was chosen since it yielded the smallest particle size of all the PEG-PCL's. The particle size of DP7 was "ideal" (31.8 to 42.6 nm) and thus have a promising potential to serve as a drug delivery system. Their viral-like size makes the micelles escape from renal exclusion in the reticulo-endothelial system and enhances their vascular permeability.

5.1.3 T_g, T_m and degradation of DP7 PEG_{5000}-PCL_{5000} and PEG_{4000}

The difference observed in T_g between the different polymers was insignificant. The DP7 yielded the lowest T_g, i.e. -31.77 °C, the PCL_{5000}-PEG_{5000} -33.34°C and PEG_{4000} -39.64 °C. This difference is quite small, so it can be estimated that it should not affect their micellization ability.

5.1.4 CAC and CMC of DP7 micelles

The CAC value for DP7 micelles was considerably lower than the CMC value (9.09 $x10^{-7}$ M and 0.245 M, respectively) as was expected. This CAC value is very low and it suggests good stability of the micelles (W. Q. Chen et al., 2008).
The CAC value is for instance much lower than CAC's for Pluronic® micelles and poly(ethyleneglycol)-distearoylphosphatidyllethanolamine polymeric micelles (their CAC range from: 1.0 x 10^{-5} M to 1.6 x 10^{-4} M) (Sezgin, Yüksel & Baykara, 2006).

5.1.5 Comparison of methods for preparation of DP7 micelles

The methods were at first compared in two aspects: transparency and particle size.
All the methods yielded transparent solutions with small particle sizes. The smallest particle size was from the CSM but the difference in particle sizes was extremely small especially between the CSM and the TFM. All three methods induced "ideal" micelles i.e. with micelle particles less than 100 nm.
Despite the above-mentioned observations, only the TFM could be used to prepare a micelle solution when the amount of FF and SA was increased to 2 mg each. For this reason, TFM was chosen as the most suitable method for preparation of DP7 micelles.

5.2 The effect of SA on micelle efficacy

Before the onset of this work, DP7 micelles had been prepared with oleic acid. The research was stopped because it was thought that a fatty acid that is in a solid form at room temperature would be a better candidate when it comes to drug loading and reducing the drug diffusion.

Initially, some solutions were prepared with a low concentration of FF and without SA. The solutions became clear and looked promising at first. After overnight in the refrigerator the particles seemed to recrystalize. This recrystallization did not appear in the solution containing SA.

A micelle solution, prepared with CSM, containing only DP7 and ultra pure water was compared to a solution containing both FF and DP7. The solution of only DP7 contained almost 8 fold bigger particles (208 nm vs. 28.5 nm) than the micelle solution containing both. When a solution containing SA was compared to the solution containing both FF and DP7 the particle size was a bit increased in the SA containing solution (37 nm vs. 28.5 nm).

Micelle solutions prepared with the TFM showed that two solutions, one containing 20 mg of DP7 and 1.5 mg FF and the other solution containing 20 mg DP7, 2 mg of FF and 2 mf of SA contained almost the same in particle size (49.2 nm and 50.4 nm)

Overall, it was clear that the particle size of micelles containing SA were smaller than micelles without SA.

When SA is included in a micelle solution, the hydrophobic interaction between the SA and the DP7 increases, leading to a smaller particle size, indicating that the micelles formed a tightly packed hydrophobic core due to enhanced hydrophobic interactions.

When an increase in the amount of FF in the micelle solution was tried, it was only possible if the amount of SA was increased in the same proportion as FF and the TFM had to be used. The effect of SA was undeniable great regarding the drug loading efficiency of DP7 micelles.

The quantification of SA did not go as planned. Many trials in different ways were performed to try to succeed in this test. The preparations of standard curves for both SA and PA were a success. To make sure that the measurements were the right ones, standard solutions were quantified at the same time as the unknown samples. The measurements for the standard solution failed so it was impossible to estimate the

amount of SA in the unknown samples. It was thought that DP7 interfered with the measurement of SA. Hence, a standard curve containing both SA and DP7 was calculated along with a standard solution containing both SA and DP7 (Appendix 5). This experiment failed as well. It is obvious that this method can not be used to quantify the SA in DP7 micelle solutions; hence a new method has to be invented.

5.3 Isolation of "ideal" micelles from other particles in a micelle solution and characterization of DP7 micelles prepared with TFM

The threestep method using Vivaspin 2 instead of a dialysis method turned out to be an efficient and simple method to isolate the "ideal" micelle particles from other particles in the solution. The Vivaspin 2 equipment has numerous advantages; it is small, light, easy and convenient to handle.

The particle size of the "ideal" micelles was 24.5 nm which is a very small. To ensure that the particles examined were indeed micelles three tests were performed on ideal micelles i.e. of a solution that had no other particles such as monomers or big particles.

The particle size, drug entrapment of FF and particle distribution of DP7 was used to confirm the existence of micelles. In an "ideal" micelle solution average particle size was 24 nm, it contained a high amount of FF and DP7. When these results from the HPLC system and the gel filtration system are combined there is evidence to prove that the solution contained "ideal" micelles.

The solution that passed through the membrane on the Vivaspin 2 equipment did not contain any FF or DP7. From these results one can conclude that the drug, fenofibrate is incorporated in DP7micelles in the "ideal" micelle solution. Otherwise the FF and DP7 would have passed through the membrane of the Vivaspin equipment.

5.4 In vitro drug release test from fenofibrate loaded DP7 micelles

Originally the intension was to compare the drug diffusion from DP7 micelles to drug diffusion of POE-PCL micelles but since it was not possible to prepare micelles containing 2 mg of FF from PEG_{5000}-PCL_{5000} it was not done. Likewise, micelles containing SA were supposed to be compared to micelles not containing SA but for the same reason as for PEG_{5000}-PCL_{5000} this test was not done.

The drug release rate from DP7 micelles was quite fast despite the low CAC and the use of SA. The reason for this remains unclear.

One aspect is that the SA was not grafted onto DP7 but mixed with the DP7 when micelles were prepared. It is possible that there is a need for the SA to be grafted onto the DP7 to affect the release rate of drug from DP7 micelles as has been done to the (CSO-SA) micelles described before (Hu, Ren et al., 2006; Ye et al., 2008).

A comparison in drug release from micelles with SA and without SA could not be performed as mentioned before. For this reason it is impossible to say if the SA had any influence on the drug diffusion rate or not. Still, the drug diffusion rate was quite fast and future studies will reveal if a slow drug release rate can be obtained from the DP7 micelles.

5.5 Enhanced solubility of FF by DP7 micelles

DP7 enhanced the solubility of FF greatly. The solubility was increased 190 to 1500 fold d to the water solubility of FF.

These results are very promising and suggest that further research on DP7 micelles should be done.

6. CONCLUSION

The amphiphilic di-block copolymer DP7 self–aggregated spontaneously into nano-sized micelles in aqueous solution. The nanoparticles prepared from the functionalized block copolymer had a very low CAC value which indicates a good stability of the micelles. The drug entrapment efficiency of the micelles was satisfying and was enhanced greatly with the inclusion of SA as the solubility of fenofibrate was increased 190 to 1500 fold compared to its water solubility.

The small size of the polymeric micelles of DP7, the very low concentration of micellization onset and the absence of toxic effects represent promising characteristics for the development of a novel polymeric drug carrier. The results suggest that the DP7 micelles present an excellent candidate for a drug delivery system.

There is still much work to be done regarding the micelles such as investigating their their activity/ and efficacy *in vivo.*

In this thesis it has been discussed that DP7 micelles are a feasible choice to enhance the solubility of hydrophobic drugs and hence can be a new approach in the struggle to find better carriers for drugs that are virtually insoluble in water.

Future studies will determine if the DP7 micelles are able to provide sustained drug release and whether they can be used in drug targeting.

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