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Electrospun amphiphilic nanofibers for the *in situ* preparation and delivery of drug-loaded liposomes

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Abstract

The physical stability is crucial in formulating liposomes for drug delivery applications. The objective of the present study was to develop and evaluate the electrospun amphiphilic nanofibers intended for the templates and delivery of drug-loaded liposomes. This approach exploits the hydration of phospholipids deposited on electrospun nanofibers for the formation of liposomes, and the strategy was recently introduced in the literature for non-pharmaceutical applications. The present study was carried out in 2014-2015 as a research collaboration between Department of Pharmacy, Faculty of Health Sciences, University of Tromsø The Arctic University of Norway, Norway and Department of Pharmacy, Faculty of Medicine, University of Tartu, Estonia.

The drug-loaded amphiphilic nanofibers were prepared by using a ESR200RD robotized electrospinning system (NanoNC, Korea). Polyvinylpyrrolidone (PVP 90) and soybean phosphatidylcholine (PC) were used at different concentrations as a filament-forming matrix. Chloramphenicol (CAM) was used at different concentrations as a model drug. The liposome formation was monitored *in situ* by using optical microscopy. The geometric properties and surface topography of nanofibers were characterized by scanning electron microscopy (SEM). Particle size of liposomes was measured by photon correlation spectroscopy (PCS). Rhodamine-labelled nanofiber-hydrated liposomes were examined using fluorescence microscopy. Drug encapsulation efficiency was determined by using high performance liquid chromatography (HPLC).

Electrospinning of PC with PVP using ethanol as a solvent was found to be successful in fabricating drug-loaded amphiphilic composite nanofibers. According to the optical microscopy results, the immediate hydration of phospholipids deposited on the amphiphilic nanofibers occurred within few seconds resulting in the formation of liposomes in water. The liposomes appeared to aggregate more readily in the concentrated solutions than in the diluted solutions. Rhodamine-labelling together with fluorescence microscopy were proved

useful techniques to illustrate the formation of liposomes. HPLC analysis indicated that the drug entrapment efficiency varied when different CAM concentrations were used.

In conclusion, it is possible to prepare drug-loaded liposomes *in situ* using the hydration of phospholipids deposited on the electrospun amphiphilic nanofibers. The present nanotechnology self-assembly approach opens up new options for the fabrication, stabilization and delivery of drug-loaded liposomes.

Sammendrag

Den fysikalske stabiliteten er essensiell i formulering av liposomer for bruk innen drug delivery. Hensikten med denne studien var å utvikle og evaluere elektrospunnete nanofibre tiltenkt som templat i produksjon av liposomer med inkorporert virkestoff. Denne metoden, nylig introdusert i litteraturen for ikke-farmasøytiske applikasjoner, benytter hydrering av fosfolipider avsatt på elektrospunnete nanofibre for å danne liposomer. Studien som danner grunnlag for denne masteroppgaven ble utført i 2014-2015 som et vitenskapelig samarbeid mellom Institutt for farmasi, Det helsevitenskapelige fakultet, Universitetet i Tromsø, Norges arktiske universitet, Norge og Department of Pharmacy, Faculty of Medicine, University of Tartu, Estland.

De amfifile nanofibrene med virkestoff ble dannet ved hjelp av et ESR200RD robotisert elektrospinningsystem (NanoNC, Korea). Polyvinylpyrrolidon (PVP 90) og fosfatidylkolin isolert fra soyabønner (PC) ble brukt i forskjellige konsentrasjoner som en filamentformende matriks. Kloramfenikol (CAM) ble tilsatt i varierende konsentrasjoner som modellsubstans. De liposomale formuleringene ble monitorert *in situ* ved bruk av lysmikroskopi. De geometriske egenskapene og overflatetopografien til nanofibrene ble karakterisert ved hjelp av scanning electron microscopy (SEM). Partikkelstørrelsen til liposomene ble målt ved hjelp av fotonkorrelasjonspektroskopi (PCS). Rodamin-merkede nanofiber-hydrerte liposomer ble undersøkt med fluorosensmikroskopi. Inkorporeringsgrad av virkestoff ble bestemt ved hjelp av high performance liquid chromatography (HPLC).

Elektrospinning av PVP og PC oppløst i etanol resulterte i vellykket produksjon av amfifile nanofibre med virkestoff. I følge resultatene fra lysmikroskopi inntraff den umiddelbare hydreringen av fosfolipidene på de amfifile nanofibrene i løpet av få sekunder og resulterte i dannelse av liposomer. Liposomene hadde en større tendens til å aggregere i de konsentrerte dispersjonene sammenlignet med de fortynnede dispersjonene. Rodaminmerking sammen med fluorosensmikroskopi var en nyttig metode for å illustrere formasjonen av liposomer. HPLC-analysene viste at inkorporering av virkestoff varierte med forskjellige konsentrasjoner av CAM.

Det kan dermed konkluderes med at det er mulig å danne liposomer med inkorporert virkemiddel *in situ* ved hjelp av fosfolipider avsatt på elektrospunnete amfifile nanofibre. Denne studien åpner nye muligheter for produksjon, stabilisering og delivery av liposomer med inkorporert virkestoff.

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List of Abbreviations

¹³ P-NMR	Phosphorous-31 nuclear magnetic resonance
AFM	Atomic force microscopy
BET	Brunauer, Emmett and Teller
CAM	Chloramphenicol
CLSM	Confocal laser scanning microscopy
EtOH	Ethanol
ES	Electrospinning
ESEM	Environmental scanning electron microscopy
Fiber-HL	Fiber-hydrated liposomes
Film-HL	Film-hydrated liposomes
HPLC	High-performance liquid chromatography
LUV	Large unilamellar vesicles
MeOH	Methanol
MLV	Large multilamellar vesicles
PC	Phosphatidylcholine
PCS	Photon correlation spectroscopy
PVP	Polyvinylpyrrolidone
SEM	Scanning electron microscopy
SUV	Small unilamellar vesicles
TEM	Transmission electron microscopy

1 Introduction

Spherical vesicles composed of phospholipids with an aqueous core, also known as liposomes, have played a big part in nanomedicine drug delivery research and product development. One of the biggest challenges when it comes to liposome preparation is to get a product which has a monodisperse population, even vesicle sizes and a decent stability (Yu et al., 2009).

Recently, Yu and coworkers (2012) prepared for the first time ever monodisperse liposomes by hydrating phospholipids deposited on electrospun amphiphilic nanofibers, composed of the hydrophilic polymer polyvinylpyrrolidone (PVP) and phosphatidylcholine (PC) (Yu et al., 2012). The templating and confinement properties of the nanofibers enabled spontaneous self-assembly of phosphatidylcholine, and liposomes were formed.

To date, liposomes self-assembled from hydrated amphiphilic nanofibers have not been exploited to fabricate drug-loaded liposomes, nor compared to any conventional liposome preparation methods.

The main aim of the present thesis was to find out whether it is possible to prepare the drug-loaded liposomes using a novel strategy based on the hydration of phospholipids deposited on electrospun amphiphilic nanofibers. In addition, the goal of the work was to find out if it is possible to improve the encapsulation efficiency, stability and size distribution of liposomes.

2 Literature review

2.1 Liposomes

2.1.1 Definition and background

Liposomes are spherical vesicles consisting of an aqueous core surrounded by one (Fig. 1) or several phospholipid bilayers. They are small in size, ranging from 50 nm to approximately 1 μm and larger (Banerjee, 2001). Liposomes are used as drug delivery vehicles, among other applications. When loading the liposomes with drug, hydrophilic molecules will be entrapped in the core of the spherical vesicle, while lipophilic drug molecules mainly will be entrapped in the fatty bilayer membrane (Laouini et al., 2012).

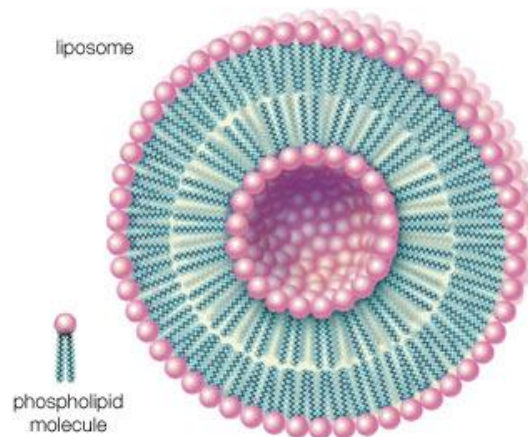


Figure 1. A single-lamellar liposome and a phospholipid molecule, which is the bilayers building block. The pink head of the phospholipid molecule illustrate the polar part, while the two blue tails illustrate the lipophilic part. Illustration: Encyclopædia Britannica, Inc., 2007.

Phospholipids self-assemble into liposomes when they are hydrated to avoid the lipophilic parts of the molecule to get in contact with water. The properties of liposomes can be manipulated by using different types of phospholipids in the bilayer, and/or by changing the surface charge, liposome size and liposome preparation (Vemuri and Rhodes, 1995). The phospholipid bilayers share a lot of the same biological properties as the plasma membranes

in our bodies (e.g. osmotic swelling, ion discrimination), thus liposomes can serve as a good model for human cell membranes (Fig. 2) (Sessa and Weissmann, 1968, Chatterjee and Agarwal, 1988).

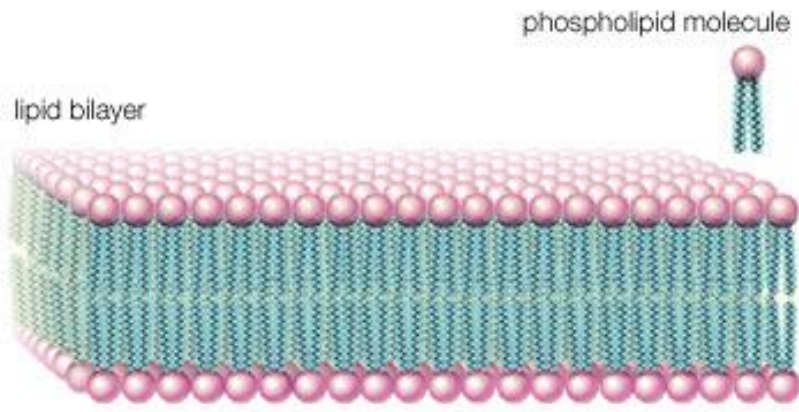


Figure 2. A schematic representation of phospholipid bilayer. The phospholipid molecules are assembled as the lipid bilayer in both liposomes and the human cell membranes. Illustration: Encyclopædia Britannica, Inc., 2007.

2.1.2 Phospholipids

Phospholipids are a group of lipids containing groups of phosphoric acid. One of the main groups of phospholipids is lecithin, which is commonly found in plants and animals, e.g. in eggs (Store Norske Leksikon, 2009).

Phospholipids are naturally occurring amphiphilic lipids. They have the ability to form lipid bilayers, partly due to their amphiphilic properties, and are an important constituent of the cell membranes in our body (Singer and Nicolson, 1972)

Phosphatidylcholine (PC) was first mentioned in 1847 as a component of egg yolk, and at that time named lecithin (Cole et al., 2012). Under today's definition, lecithin is a mixture of a variety of phospholipids and one of these lipids is PC (Martindale, 2013), but the name lecithin is still sometimes used as a synonym.

PC consists of a glycerol backbone with three groups attached to it (Fig. 3). Two of these are fatty acids, connected by ester bonds. Choline is the third group, attached by a

phosphodiester linkage. The length and double bonding of the fatty acyl chains will vary in different PC molecules (Cole et al., 2012).

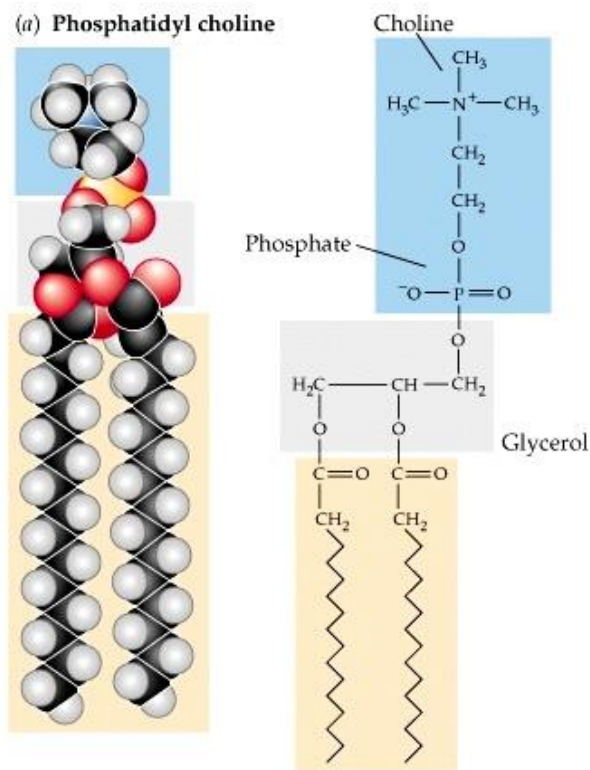


Figure 3. The chemical structure of a phosphatidylcholine (PC) molecule. The glycerol backbone is marked grey, while the choline is blue and the fatty acyl chains are marked orange. Figure from Purves et al., 2003: *Life, Science of Biology*, Sixth Edition, by Sinauer Associates, Inc.

2.1.3 Classification of liposomes

Liposomes are classified according to a wide variety of factors – like their size, lamellarity, application, surface charge and production method. In this section, the main focus is on size, lamellarity and surface properties.

There are three different classes of liposomes divided based on their size and lamellarity (Banerjee, 2001) (Fig. 4):

1. Small unilamellar vesicles (SUV), with one lipid bilayer and diameter size 25-100 nm;
2. Large unilamellar vesicles (LUV), with one lipid bilayer and diameter size 100-1000 nm;
3. Large multilamellar vesicles (MLV), with several lipid bilayers and diameter size over 1000 nm.

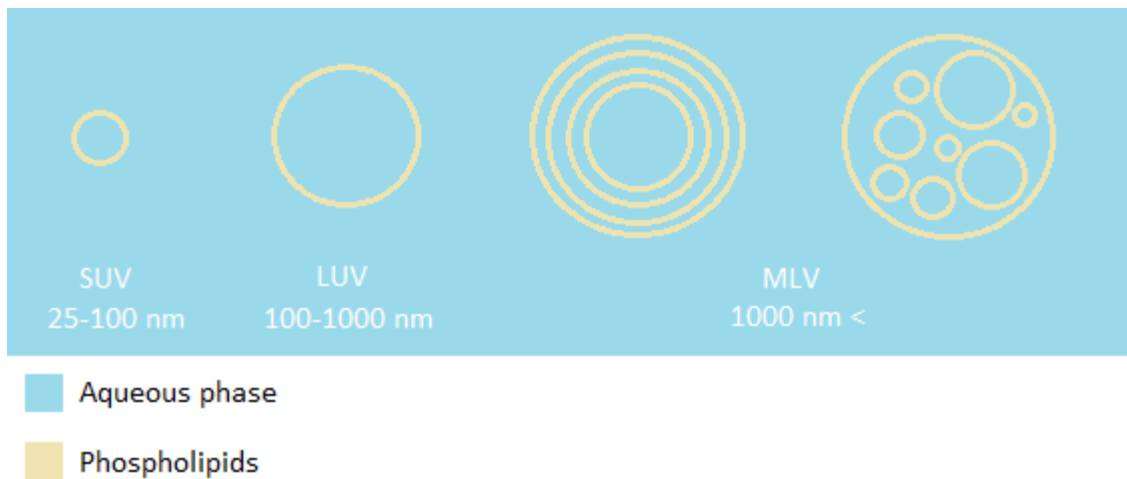


Figure 4. The three main types of liposome classes and their size range given in diameter (nm). Small unilamellar vesicles (SUV) are the smallest liposomes, consisting of one bilayer and with a diameter ranging from 25-100 nm. Large unilamellar vesicles (LUV) have the same structure, but are a bit larger: 100-1000 nm. There are two types of large multilamellar vesicles (MLV) depending on how the bilayers are arranged inside the outer shell. MLV's are the biggest group of liposomes, 1000 nm and larger.

Liposomes can also be classified based on their surface properties, and this type of classification is naturally more commonly used when dealing with liposomes for *in vivo* use. The way it is possible to manipulate the surface of liposomes gives them a great advantage when compared to the other colloidal drug delivery systems (Storm and Crommelin, 1998).

There are four main groups:

1. *Conventional liposomes*. Typically made from phospholipids and/or cholesterol. They can be neutral or negatively charged. Their size and lamellarity (Fig. 4) can easily be manipulated. Even though these conventional liposomes can be prepared differently, they still have about the same characteristics when injected into the bloodstream. They tend to accumulate and are broken down in the mononuclear phagocyte system (MPS), which makes the blood circulation time short compared to other liposomes.
2. *Long-circulating liposomes*. These liposomes are made so that they will be circulating in the bloodstream for a relatively longer time before being taken up by the MPS. The most common way of making long-circulating liposomes is by covalently attaching hydrophilic polymer polyethylene glycol (PEG) on the outside of the lipid bilayer (Fig. 5). These types of long-circulating liposomes are called stealth liposomes.

3. *Immunoliposomes*. They have antibodies or antibody fragments attached to their shell. These liposomes can be combined with PEGylation to increase the time of circulation in the bloodstream.
4. *Cationic liposomes*. These liposomes have advantages when it comes to the delivery of genetic material. The positively charged surface interacts and neutralizes negatively charged DNA (Storm and Crommelin, 1998).

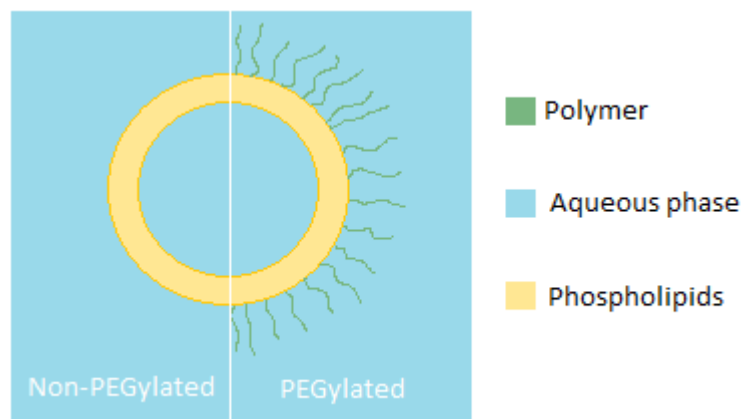


Figure 5. Illustration showing a non-PEGylated and a PEGylated surface of a liposome.

2.1.4 Characterization of liposomes

Liposome physiochemical properties like lamellarity, surface charge, shape and size are very much influencing the behavior of the vesicles, e.g. how rapidly they are cleared from the bloodstream (Juliano and Stamp, 1975) . Characterization of the liposomes is important to understand and predict how the liposomes will act in prospective applications.

Photon correlation spectroscopy (PCS) is a powerful analytical technique that can be used to determine the vesicle size and size distribution of a liposome dispersion. The PCS shows an insensitivity towards very small vesicles (<30 nm) if larger particles are present in the sample, and therefore is most often used combined with a size reduction of the liposomes prior to analysis (Ingebrigtsen and Brandl, 2002).

Microscopic characterization methods like atomic force microscopy (AFM), environmental scanning electron microscopy (ESEM), transmission electron microscopy (TEM), and confocal

laser scanning microscopy (CLSM) can be used to study the shape, morphology, dimensions, surface properties and internal structure of the liposomes. Scanning electron microscopy (SEM) requires the sample to be dried or fixed before imaging, thus this is not an applicable method for the characterization of liposomes due to risk of liposomal damage (Karn et al., 2013).

The way the liposomes encapsulate drug, how the drug is released and the intracellular fate of the liposomes are all factors affected by the liposome lamellarity. Phosphorous-31 nuclear magnetic resonance (^{31}P -NMR) spectroscopy is a widely spread method used to characterize the liposomes which shows how many phospholipid bilayers the liposomes have and how they are arranged compared to each other (Fröhlich et al., 2001).

The amount of drug which is trapped inside the aqueous core or the lipid bilayer of the liposomes is commonly calculated using a high performance liquid chromatography (HPLC). Drug encapsulation efficiency can be determined by using HPLC and analyzing how much drug that has been trapped inside the vesicles compared to the amount of drug which has not been encapsulated (Edwards and Baeumner, 2006).

2.1.5 Liposome preparation methods

There are many different ways of producing liposomes, and the method used can affect the properties of the liposomal product. Encapsulation efficiency is one example of a property that could be influenced by the production method, as shown by Liang and coworkers. In their study, they concluded that by preparing the liposomes by freeze-dried monophasic systems hydration method, they were able to entrap a higher amount of lipopeptides than if they hydrated dried lipid films (Liang et al., 2005).

There are a variety of different ways to produce the liposomes, and these can be categorized into two major groups (Patil and Jadhav, 2014):

1. Transferring of phospholipids in an organic phase into an aqueous phase to produce liposomes known as *bulk methods*.

2. Depositing of a thin lipid film on a substrate and subsequently hydrate to form liposomes known as *film methods*.

2.1.5.1 Conventional hydration of a phospholipid film

The oldest and most common way of preparing liposomes is a thin-film hydration method. It is a simple method which does not require any advance equipment, and this is why it is so frequently used. Lipids are dispersed in an organic solvent in a round bottom flask, and the solvent is evaporated using a rotary evaporator with a lowered pressure. The dry thin-film deposited on the inside of the flask is hydrated and liposomes self-assemble (Fig. 6). Like most conventional methods, this normally yields a dispersion containing liposomes of heterogeneous size and shape, and thus a size reduction technique is a common next step (Laouini et al., 2012).

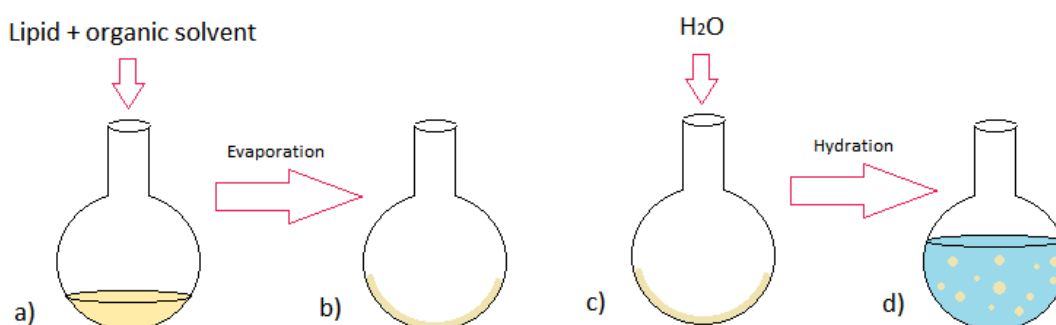


Figure 6. The basic principle of a thin-film hydration method: a) Lipid and organic solvent are mixed together in a round bottom flask; b) Solvent is evaporated and remaining is a thin lipid film deposited on the inside of the flask; c) Water, often in the form of a buffer, is added and the flask is shaken; d) The thin film is hydrated and a heterogeneous dispersion of liposomes are self-assembled.

2.1.5.2 Microfluidics method – thin film hydration

Microfluidics is the technology, which allows performing fluid procedures in a very small geometrically constrained volume. This relatively new technology opens a number of possibilities when combined with other scientific knowledge. One example is the fabrication of liposomes by exploiting these microfluidics devices (van Swaay, 2013, Patil and Jadhav, 2014).

Microfluidics is a very versatile method, which makes it possible to produce liposomes ranging from tens of nanometer to tens of micrometers in diameter (Yu et al., 2009).

2.1.6 Liposomes as drug delivery system

Liposomes have been extensively used in pharmaceutical applications and they show a great amount of favorable properties as drug delivery systems. They offer metabolic protection of the drug, higher target specificity, lower toxicity, and elongate circulation time and controlled drug release.

Administration route and mode of action determines the liposomal properties that are preferred for the drug-delivery. In the systemic bloodstream, for instance, it has been observed that liposomes from approximately 150-200 nm in diameter have a longer circulation time than the liposomes with a diameter of around 70 nm (Litzinger et al., 1994).

The liposome size is not only important for systemic drug-delivery, but also for topical application. Verma and coworkers (2003) showed that the liposomal size was inversely related to the skin penetration of liposomes loaded with the hydrophilic fluorescent labeled compound carboxyfluorescein. They observed that a vesicle size of around 120 nm in diameter showed an enhanced skin penetration (Verma et al., 2003).

2.2 Electrospun nanofibers

2.2.1 Definition and background

Fibers with a diameter under 1 μm are commonly referred to as nanofibers (Grafe and Graham, 2003). The decrease in a diameter makes the surface area very large compared to the volume of the fibers, and gives the fibers unique properties which make them optimal candidates for several uses (Huang et al., 2003).

Electrospun nanofibers have a large variety of application fields (Fig. 7). They can for instance be exploited as textiles (Gibson et al., 1999), filters (Hajra et al., 2003), tissue engineering (Li et al., 2005), for wound healing (Boland et al., 2001) and/or drug delivery (2.2.6).

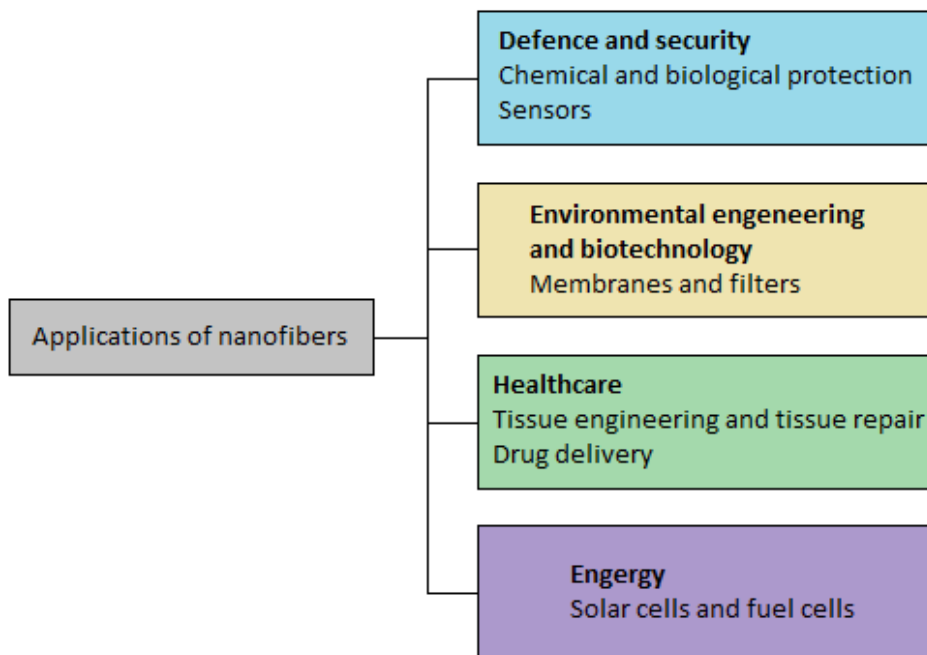


Figure 7. Potential applications for electrospun nanofibers. Adapted and modified from Ramakrishna et al. (Ramakrishna et al., 2006)

Drug release from a nanofibrous delivery system is dependent on a variety of elements like biocompatibility, mechanical properties, morphology and internal structure of the nanofibers. Nanofibers are often constructed of polymers, and preferably biodegradable polymers are used. Polymeric drug delivery systems have the ability to achieve the desired controlled-release of active drug over a long period of time (Liechty et al., 2010).

2.2.2 Preparations of nanofibers – electrospinning (ES)

A variety of nanofiber processing techniques like self-assembly (Niece et al., 2003) or phase separation (Liu and Ma, 2009) have been practiced to fabricate nanofibers. However, the ES technique is the most used and convenient method of nanofiber production. It gives a

unique opportunity to manipulate the properties such as the diameter, surface area, weight, density and orientation of the fibers (Širc et al., 2012).

During ES, high voltage is applied to a pendent drop of a polymer solution being pumped out from a syringe needle (Fig. 8). The drop will get electrically charged, and a deformation of the drop (Taylor Cone) will appear when the voltage threshold is surpassed. An electrically charged jet will form and move towards the counter electrode. The solvent will evaporate on the way and a web of ultrathin nanofibers will be formed (Huang et al., 2003, Greiner and Wendorff, 2007). This method can yield fibers with a diameter ranging from just a few nanometers up until several micrometers (Širc et al., 2012).

One can also perform ES using a melt instead of the polymer solution. Melt-electrospinning possesses the same general principle as for the ES of polymeric nanofibers, but what turns the jet into solid nanofibers is decrease in temperature, and not the evaporation of solvent as for polymer solutions (Lyons et al., 2004).

ES is also easy to use to produce nanofibers in a large-scale and cost-effective way, thus convenient when it comes to manufacturing (Persano et al., 2013). This method makes it simple to add drugs or other important components to the fibers to achieve wanted characteristics. For example, growth factors can simultaneously be loaded into the nanofibers, thus resulting in more advanced and elegant novel nanosystems (Ignatova et al., 2013).

2.2.3 ES set-up and materials for electrospinning

The ES set-up is very simple. It consists of a syringe with a polymer solution (or melt) and a metallic needle (spinneret), a high voltage power supply and a grounded collector plate (Fig. 8) (Li and Xia, 2004). The voltage is applied between the syringe needle and the collector plate.

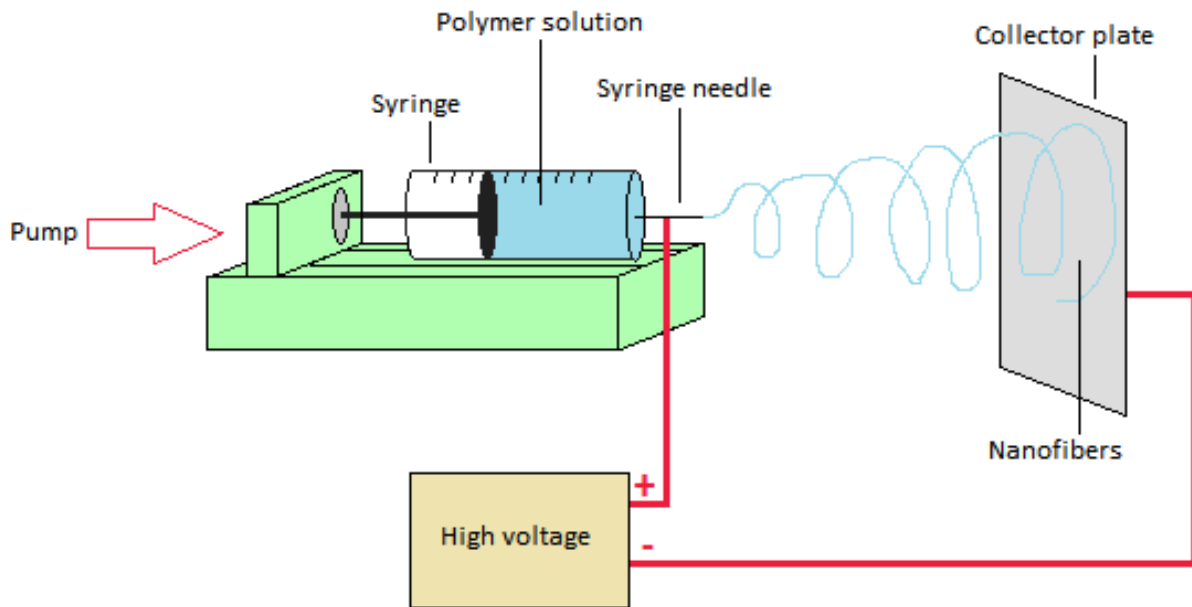


Figure 8. A schematic diagram of an electrospinning (ES) set-up. A pump pushes the polymer solution out of the syringe and through the metallic needle. The high voltage power supply forces the solution to stretch and form into nanofibers, which are gathered onto a grounded collector plate.

The materials used for ES have to undergo strong deformations and large amounts of stress to be pulled into thin nanofibers. Thus, the material used has to be viscoelastic and also cohesive at the same time to end up as fibers at the end of the process (Huang et al., 2003).

The ES process can form nanofibers from a huge range of materials like polymers, composites, semiconductors and ceramics. Nearly all soluble or meltable polymers with a sufficiently high molecular weight can be electrospun and turned into fibers if the process parameters are optimal (Greiner and Wendorff, 2007).

2.2.4 Process parameters and possible problems related to processing

The properties of the fibers are very much dependent on the process parameters during electrospinning (Li and Wang, 2013):

- Voltage. The voltage has to be higher than the threshold voltage for charged jets to form the Taylor Cone. A voltage that is too low will not be sufficient to electrospin all of the polymer solution and dripping will occur. A voltage that is too high will create

an uneven jet. It has been discussed how different voltage affect the diameter of the nanofibers, and so far the results have been different depending on the polymer used;

- Flow rate. Generally a low flow rate is recommended so that the polymer solution gets enough time to be polarized and so that the solvent gets enough time to evaporate on the way towards the collector. Dripping will occur if the flow rate is too high, and problems with an unstable jet could occur if the flow rate is too low;
- Collectors. There is a great variety of different collectors on the market, and the choice of a collector has also an influence on the ES process and the resulting fibers;
- Distance between the metal needle and the collector. The distance has to be long enough to let the solvent get enough time to evaporate on its way towards the collector. If the distance is too long, beads can form on the fibers, thus an optimum distance is recommended (Li and Wang, 2013).

2.2.5 Characterization of electrospun nanofibers

Morphology, specific surface area, volume and porosity are all important contributions to determine the nanofibers properties. Characterization is an important mean to understand how a specific nanofiber composition would work as a drug delivery system or in other applications. There are many different means of characterization, some of which are mentioned here.

Imaging methods are today widely used as a characterization method. Optical microscopy, TEM, SEM, and AFM are all used as frequent imaging techniques to investigate nanofibers. Optical microscopy is simple to use for investigating the physical appearance and quality of the nanofibers. It is a fast, simple and cheap imaging method, but has a limited resolution which makes it a problem to capture very detailed images (Širc et al., 2012).

Microscopy methods like TEM, SEM and AFM are used when more detailed information is required. Since there are several imaging methods available which can be used for characterization, the best and most often used approach is to use a combination of methods for different aspects of the evaluation. But when it is known what specific information is

needed, only one method could be applied. Amiraliyan and coworkers (2009) used SEM images to determine mean diameter of electrospun silk nanofibers. SEM images were taken, and approximately 100 random nanofibers were selected to calculate the average diameter (Amiraliyan et al., 2009). Chen and coworkers (2010) exploited TEM to verify a core-shell structure and AFM to examine surface properties in their coaxial electrospun nanofibers (Chen et al., 2010).

To measure nanofiber porosity, mercury porosimetry (Ritter and Drake, 1945) can be used. Mercury is transferred into the sample under vacuum and the porosity is calculated from the mass of mercury intruded into the pores of the nanofibrous mat.

Specific surface area can be determined using gas adsorption method and using a Brunauer, Emmett and Teller (BET) theory (Brunauer et al., 1938). The methods enable calculation of the specific surface area based on adsorbed gas volume on the surface of the sample (Širc et al., 2012).

Contact angle measurement is a common way to determine the hydrophilicity of the nanofibrous mat. Static water contact angles are measured using a sessile drop system to determine in which degree the fibers repulse the water drop (Yu et al., 2011).

2.2.6 Nanofibers as drug delivery system – wound healing

As mentioned, nanofibers have numerous applications, and one of them is their use as a drug delivery system and/or dressing for successful wound healing. The decrease in fiber diameter makes the surface area very large compared to the volume of the fibers, and gives the fibers unique properties which make them optimal candidates for several applications, included wound healing (Huang et al., 2003). ES nanofibers have several other characteristics that favour their use in such applications including an ability to mimic the fibrillary structure of natural extracellular matrix, interconnecting porous structure with high permeability, and the ability to incorporate active pharmaceutical ingredients (Hu et al., 2014). As an example, the preparation of nanofibrous wound dressings consisting of silver as an antimicrobial agent have been introduced. Nanoparticles of silver can be easily deposited on nanofibers

and the antibacterial effect of the silver will be well contained (Rai et al., 2009). Therefore, this example illustrated how the use of nanotechnology and nanofibers improved the properties of silver important for sufficient wound healing.

There are many types of wounds, but the ideal conditions for wound healing will roughly be the same. Wound healing is being carried out best in a moist environment, since both an excess as well as insufficient exudate production may prolong the wound healing (Leaper et al., 2012). The regenerating cells and tissue need a good circulation of oxygen as well. The material covering the wound should be protecting it from outside bacteria, but still be permeable to oxygen and moisture to optimize the healing process. The big diversity of wounds has resulted in many different targets in wound therapy and thus a wide assortment of wound dressings. A sufficient dressing should be at low cost, optimize healing conditions and be as comfortable as possible for the user (Boateng et al., 2008). Preferably, also antimicrobial agents could be included within the dressing that would decrease the microbial bioburden and biofilm formation (Rhoads et al., 2008, Leaper et al., 2012). It is important that the material used keeps the skin damp, but still enables absorption of moisture to prevent exudates from accumulating, because this accumulation can increase the chance of an infection developing (Khil et al., 2003).

Electrospun nanofiber mats have some distinctive qualities that could make them suitable for wound healing. Their high specific surface area and small pores makes them advantageous for absorbing liquid and protecting against bacteria. The materials must be chosen carefully to achieve these wanted effects (Khil et al., 2003, Rathinamoorthy, 2012)

Core-shell structured nanofibers can be fabricated using coaxial electrospinning. The coaxial ES apparatus has, in contrary to the standard ES apparatus, *two* capillaries placed together coaxially. A core solution is injected through the inner needle, and a shell solution, most often consisting of a spinnable polymer, is injected through the outer needle (Mickova et al., 2012). This leads to second-generation nanofibers with a core-shell structure. The core solution can for instance be a liposome dispersion and the system can be used for prolonged drug delivery (Mickova et al., 2012).

2.3 Combining liposomes and ES – novel methods for liposome preparation

Today there are several conventional and novel methods used for liposome preparation, and they all have their advantages and disadvantages (Patil and Jadhav, 2014). Some of these new methods include ES in the process of fabrication.

When a polymer with a shorter chain is used for ES, as a result an electrospray instead of the nanofibers will be formed as a final product. This technology has been exploited to fabricate liposomes loaded with naproxen (Yu et al., 2012).

Song and coworkers fabricated liposomes made from hydrated electrospun fibers comprising PVP, PC and Fe_3O_4 nanoparticles. They found the liposomes to be generally well dispersed, stable to generation and they were able to control the vesicle size with the variation of Fe_3O_4 in the fibers (Song et al., 2014)

Recently, Yu and coworkers (2011) introduced a novel strategy for using electrospun composite nanofibers as templates in fabricating the liposomes. They described how the prepared electrospun nanofibers composited of different concentrations of a polymer and a phospholipid, and how the liposomes self-assembled after the hydration of these fibers (Yu et al., 2011).

3 Aims of the study

The main objective of the present study was two-fold:

- (i) To prepare and characterize the drug-loaded liposomes using conventional film hydration method and novel hydration of phospholipids deposited on electrospun amphiphilic nanofibers.
- (ii) To compare the relevant properties of the prepared liposomes.

Together with this main goal, also an important specific aim was to prepare the amphiphilic nanofibers by using ES and characterize their main physiochemical properties.

4 Materials and methods

4.1 Materials

Chloramphenicol (Sigma-Aldrich, Chemie GmbH, St. Louis, USA; Lots SLBH3546V and 120M0175V) was used as an antibiotic. Soybean phosphatidylcholine (Lipoid S-100, Lipoid GmbH, Ludwigshafen, Germany) was used as a basic material for preparing the liposomes. Polyvinylpyrrolidone, PVP (Kollidon 90F K90, BASF SE, Germany; Lot 82296056PO) was applied as a carrier polymer in electrospinning (ES). Rhodamine 123 (Sigma-Aldrich, Chemie HmbH, St. Louis, USA; Lot BCBL8890V) was used as an auto-fluorescent marker in the fluorescence microscopy studies. Other solvents (ethanol, 96.5 %, methanol) were of analytical grade obtained from Sigma).

4.2 Preliminary tests

4.2.1 Materials selection for ES of nanofibers

The material selection for the present study was based on the original work published by Yu and coworkers (2011). They fabricated amphiphilic nanofibers composed of the mixture of PVP K60 and soy bean PC using chloroform as an organic solvent in ES. In the current study, an extensive number of pre-tests were carried out to find a proper composition of ingredients that could be used for successful ES. On the contrary to Yu et al. study (2011), PVP K60 was replaced with PVP K90 and EtOH was used as an organic solvent instead of chloroform due to its accessibility, safety and practical matters. Two different polymer concentrations (5% and 6% w/v PVP in EtOH) were tested for ES in order to find a polymer concentration that made the ES process easy to perform and gave adequate nanofibers (Table 1). Afterwards, different concentrations of PC (20% and 33.3%) and CAM (4% and 20%) were added in the nanofibers to determine how this affected the nanofiber formation and if it was executable to prepare the fibers with these compositions.

Table 1: Nanofiber compositions

Nanofiber	CAM (w/w% of the fibers)	PC (w/w% of the fibers)	PVP (w/w% of the fibers)
nf1	-	-	100
nf2	-	20	80
nf3	-	33.3	66.7
nf4	4	32	64
nf5	20	32	48

4.2.2 Process parameters selection

The significant process parameters (injection rate, voltage provided, size of needle etc.) of the ES system were varied to identify and find out which settings would give the most stable and problem-free ES process (4.2.1).

4.3 Preparation methods

4.3.1 Preparation of electrospun amphiphilic nanofibers

4.3.1.1 ES set-up

The amphiphilic nanofibers were fabricated with an ESR200RD robotized ES system (NanoNC, Korea). The ES process was carried out using a 2.5 ml syringe with a 25G needle and an injection rate of 5 ml/h, provided by an automatic syringe pump. The voltage was varied between 10-12 kV. The distance between the needle and the collector plate was approximately 11 cm. The fibers were electrospun in an atmosphere of 18-20% air humidity and at temperatures of 22-25°C.

4.3.1.2 ES protocol and experiments

Nanofibers of five different compositions (Table 1) were fabricated for this study using the set-up elaborated in section 4.3.2.

To prepare the solutions for ES, PVP (0.6 g) was dissolved in 96.5 % (w/v) EtOH (9.06-9.28ml). Most of the nanofibers were made with phospholipids and the model drug, and in these cases the PC (0.12 g or 0.3 g) and CAM (0.0375 g or 0.125 g) were dissolved in EtOH before the polymer was added. The solution was pulled up in a syringe and the syringe was attached to the ES set-up. The voltage was adjusted during ES to keep a stable jet and avoid dripping.

Three types of nanofibers were electrospun (Fig. 9):

- a) Polymeric nanofibers (100% PVP)
- b) Amphiphilic nanofibers (PC + PVP)
- c) Drug-loaded amphiphilic nanofibers (CAM + PC + PVP)

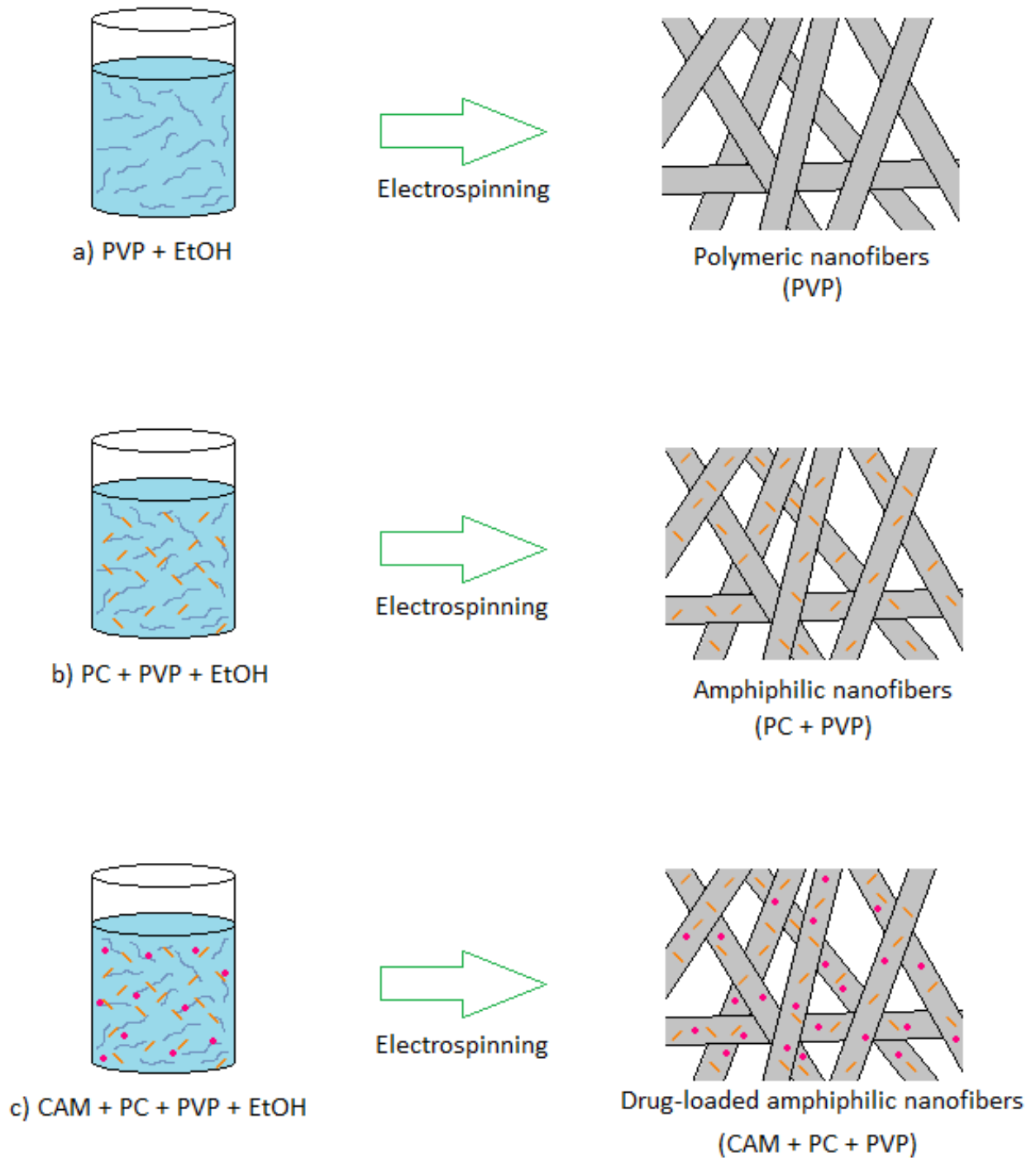


Figure 9. Schematic representation of prepared nanofibers and their compositions; a) Polyvinylpyrrolidone K90 (PVP) is dissolved in ethanol (EtOH) and electrospun to form polymeric nanofibers; b) Phosphatidylcholine and PVP are dissolved in EtOH and electrospun to form amphiphilic nanofibers; c) Chloramphenicol (CAM), PC and PVP are dissolved in EtOH and electrospun to form drug-loaded amphiphilic nanofibers. EtOH will evaporate during the electrospinning process, and the other components of the original solution will form the nanofibers.

4.3.2 Preparation of liposomes

4.3.2.1 Preparation of fiber-hydrated liposomes (fiber-HL)

Total of 100 mg nanofibers (nf3, nf4 or nf5) (Table 1) were hydrated with 1 ml distilled water and manually shaken (1-2 min) until the nanofibers were dissolved and a white and homogenous dispersion was obtained (Table 2) (Fig. 10). Reproducibility of the liposome preparation was confirmed using replicates.

Table 2: Composition of the fiber-HL dispersions

Liposome dispersion	Hydrated nanofiber (100 mg)	H ₂ O (ml)	CAM/PC (%)
Fiber-HL0	nf3	1	- *
Fiber-HL1	nf4	1-5	12,5
Fiber-HL2	nf5	1-5	62.5

*Liposome dispersion does not contain CAM

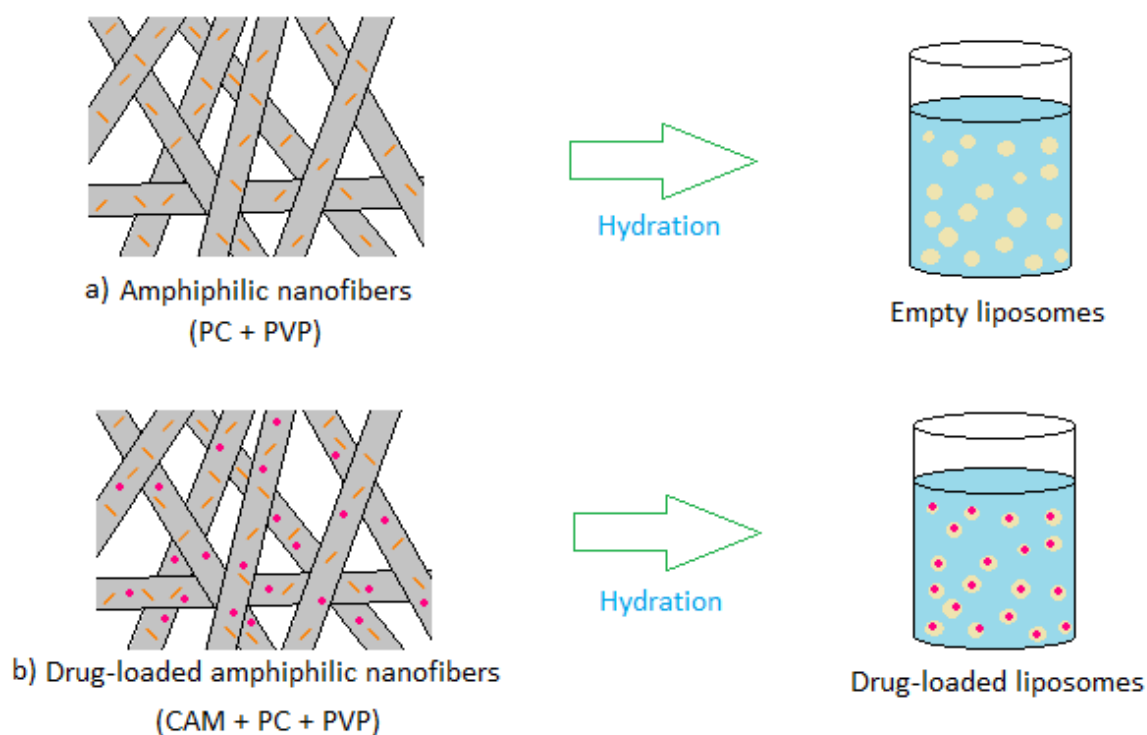


Figure 10. Schematic representation of prepared liposome dispersions from hydrated nanofibers, a) Amphiphilic nanofibers consisting of phosphatidylcholine (PC) and polyvinylpyrrolidone (PVP) are hydrated to form empty liposomes; b) Drug-loaded amphiphilic nanofibers consisting of chloramphenicol (CAM), PC and PVP are hydrated to form drug-loaded liposomes.

4.3.2.2 Preparation of film-hydrated liposomes (film-HL)

Liposomes with two different CAM-concentrations (14.3% and 62.5% CAM/PC) (Table 3) were prepared using the thin-film hydration method for later comparison to the fiber-HL. The drug:lipid ratio in the film-HL were targeted to approximately match the ratio in the fiber-HL (4.3.2.1) made from hydrated nf4 and nf4 (Table 1). CAM (28.6 mg or 125 mg) and PC (200 mg) were dissolved in EtOH (20 ml) in a round bottom flask. The EtOH was evaporated using a rotary evaporator for 20 min at 150 mbar (45 °C and 80 rpm), and subsequently for 1 h at 50 mbar (45 °C and 80 rpm). The time period was extended if needed. After the thin lipid film was dry, 10 ml of distilled water was added and the round bottom flask and its contents were manually shaken for approximately 20 min. Vortex was used if needed. Reproducibility of the liposome preparation method was confirmed using replicates (n=2).

Table 3: Composition of the film-hydrated liposome (film-HL) dispersions

Liposome dispersion	CAM (mg)	PC (mg)	H ₂ O (ml)	CAM/PC (%)
Film-HL1	28.6	200	10	14.3
Film-HL2	125	200	10	62.5

4.4 Characterization of electrospun amphiphilic nanofibers

4.4.1 Optical microscopy

The electrospun nanofibers (4.3.1) were magnified and imaged using a optical microscope CETI MAGTEX (Medline Sci.).

4.4.2 Scanning electron microscopy (SEM)

Samples of the electrospun nanofibers nf1-4 (4.3.1) were magnified and imaged using a high-resolution scanning electron microscope (SEM, Zeiss EVO MA, Germany). Both the diameter and surface morphology of nanofibers was investigated (4.6.1). Samples were

mounted on aluminum stubs with silver paint and magnetron sputter coated with a 3-nm gold layer in argon atmosphere prior to SEM microscopy.

4.5 Characterization of fiber-hydrated (fiber-HL) and film-hydrated liposomes (film-HL)

4.5.1 Ultracentrifugation of liposome dispersions

To further characterize and compare differently prepared liposomes using fluorescence microscopy and high-performance liquid chromatography (HPLC), the liposome dispersions (fiber-HL1-2 and film-HL1-2) were centrifuged in a Beckman Coulter ultracentrifuge with a SW55 Rotor at 55 000 rpm (for 1 h at 4 °C) to prepare more concentrated dispersions.

Since the work was performed in different laboratories, the liposome dispersions to be characterized by the PCS method were centrifuged in a Beckman Optima LE-80K with a SW50.1 rotor at 50 000 rpm (for 1 h at 4 °C).

4.5.2 Optical microscopy

Concentrated and not concentrated dispersions of the fiber-HL (4.3.2.1) were magnified and imaged using an optical microscope CETI MAGTEX (Medline Sci.). The concentrated fiber-HL was made by self-deposition in a vacuum for 10 min.

4.5.3 Fluorescence microscopy

Fluorescence microscopy was used to visualize the liposomes and investigate their morphology. For liposome imaging, rhodamine 123 (0.0010 g) was dissolved in distilled water (250 ml) (Fig. 11).

4.5.3.1 Film-HL fluorescence microscopy

Liposome dispersion (5 ml) (film-HL1 and film-HL2) was centrifuged (4.5.1). Liposome pellet was resuspended in a rhodamine 123 solution (1 ml) and incubated for at least 1 h before imaging with Fluorescence Microscope System (DM 5500 B, Leica Microsystems).

4.5.3.2 Fiber-HL fluorescence microscopy

Total 75 mg nanofibers (nf4 and nf5) (Table 1) were hydrated with 1 ml rhodamine 123 solution, incubated at least for 1 h at room temperature and imaged with Fluorescence Microscope System (DM 5500 B, Leica Microsystems).



Figure 11. Picture of rhodamine 123 solution used for labelling of the liposomes

4.5.4 **Particle size analysis – photon correlation spectroscopy (PCS)**

The PCS method was used to analyze the particle size and particle size distribution of the liposomes. The preparation of the samples was performed in a laminar flow cabinet to prevent contamination. The test tubes were put in distilled water and sonicated for 10 min in an ultrasonic bath to remove the dust particles before use, and subsequently they were put in the laminar flow cabinet and washed with distilled and freshly filtered water. One small drop of film-HL (4.3.2.2) or fiber-HL (4.3.2.2) was added to the tube before being diluted with distilled and freshly filtered water. The sample was diluted until the particle count on the display of the instrument was within the range of 250-350 KHz (Ingebrigtsen and Brandl, 2002). The particle size analysis was performed at 24-25 °C. All measurements

were performed in triplicates. Each liposome dispersion was analyzed 3 times in measurement cycles of 10 min. To prevent any possible interference with the results, the fiber-HL was ultracentrifuged (4.5.1) before PCS analysis and the supernatant containing polymer was removed.

All measurements were performed on the PCS instrument Nicomp submicron particle analyzer model 380, Nicomp Inst Corp.

4.5.5 Drug encapsulation efficiency – high-performance liquid chromatography (HPLC)

Film-HL (4.3.2.2) (5 ml) and fiber-HL (4.3.2.1) (5 ml) were ultracentrifuged (4.5.1). Both supernatant and pellet were analyzed by HPLC. The mobile phase consisted of 20% phosphoric acid, 100% methanol (MeOH) and water in the ratio of 5:40:55. The detector wavelength was set to 275 nm and a C18 standard column was used. The sample was diluted in MeOH prior to analysis. All measurements were performed in duplicates.

To calculate the encapsulation efficiency, the following equation was used:

$$\text{Encapsulation efficiency (\%)} = \frac{W_{\text{pellet}}}{W_{\text{total}}} \cdot 100,$$

Where;

W_{pellet} = amount of CAM in the pellet

W_{total} = amount of CAM in the whole sample (supernatant + pellet)

4.6 Data analysis

4.6.1 Diameter measurement of nanofibers

The SEM-images (4.4.2) were analyzed using the image processing computer program ImageJ to calculate a diameter mean of each of the fiber compositions. The bar scale was used to calibrate the computer program, and diameters of randomly measured 100 nanofibers were measured to calculate a mean diameter.

4.6.2 PCS results interpretations

4.6.2.1 For Gaussian distribution

A Gaussian distribution mode was applied for measurements where the value for Chi-squared did not exceed 3.00 (Ingebrigtsen and Brandl, 2002). A mean value was calculated for such samples.

4.6.2.2 For Nicomb distribution

A Nicomb distribution mode was applied for measurements where the value for Chi-squared exceeded 3.00 (Ingebrigtsen and Brandl, 2002). One measurement from each of the analyzed liposome dispersions (with Chi-sq>3) was chosen as a representative and the results were presented in tables.

Intensity weighted distribution was used for both Gaussian and Nicomb distribution.

4.6.2.3 Statistical evaluations

When applicable, calculation of mean, standard deviation and t-test were performed using Microsoft Excel 2010.

5 Results and discussion

5.1 Characterization of electrospun amphiphilic nanofibers

5.1.1 Optical microscopy of nanofibers

Optical microscopy images of aluminum foil and nf1-nf3 (Table 1) are shown in Figure 12. The images show that nanofibers consisting of 100% PVP (b), 20% PC/80% PVP (c) and 33.3% PC/66.7% PVP (d) were successfully electrospun without any visible defects. It has been reported that the processing defects (beads) may be present when the processing conditions and/or the solvent properties are not optimized (Fong et al., 1999). Therefore, it can be concluded that the viscosity and surface tension of the polymer solution was close to optimal.

Aluminum foil was imaged separately (Fig. 12a) to help to interpret the results and so that this background could be distinguished from nanofibers. All the nanofibers were electrospun and collected on aluminum foil, and the presence of foil is still visible due to thin-layer nanofibers (Fig. 12b).

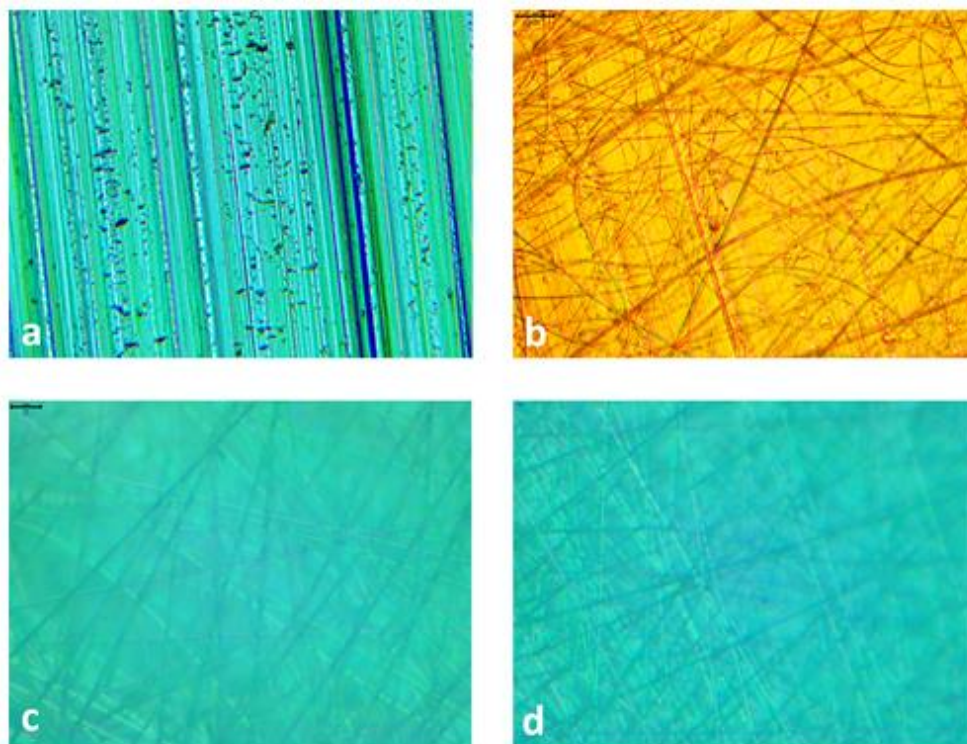


Figure 12. Optical microscopy images showing aluminum foil (background) and the morphology and structure of three different composite nanofibers; a) Aluminum foil (X50), blue filter; b) nf1 (X50), yellow filter, scale bar in top left corner corresponds to 10 μm ; c) nf2 (X40), blue filter, scale bar in top left corner corresponds to 10 μm ; d) nf3 (X40), blue filter

5.1.2 Scanning electron microscopy (SEM)

SEM micrographs of the amphiphilic nanofibers nf1-nf4 (Table 1) are shown in Figure 13. The micrographs show a nonwoven pattern, a smooth surface and an absence of beads in all of the investigated nanofibers – thus verifying the optical microscopy results.

There is no visible dissimilarity detectable regarding the surface topography or geometric properties when comparing the SEM micrographs of these four different nanofiber compositions.

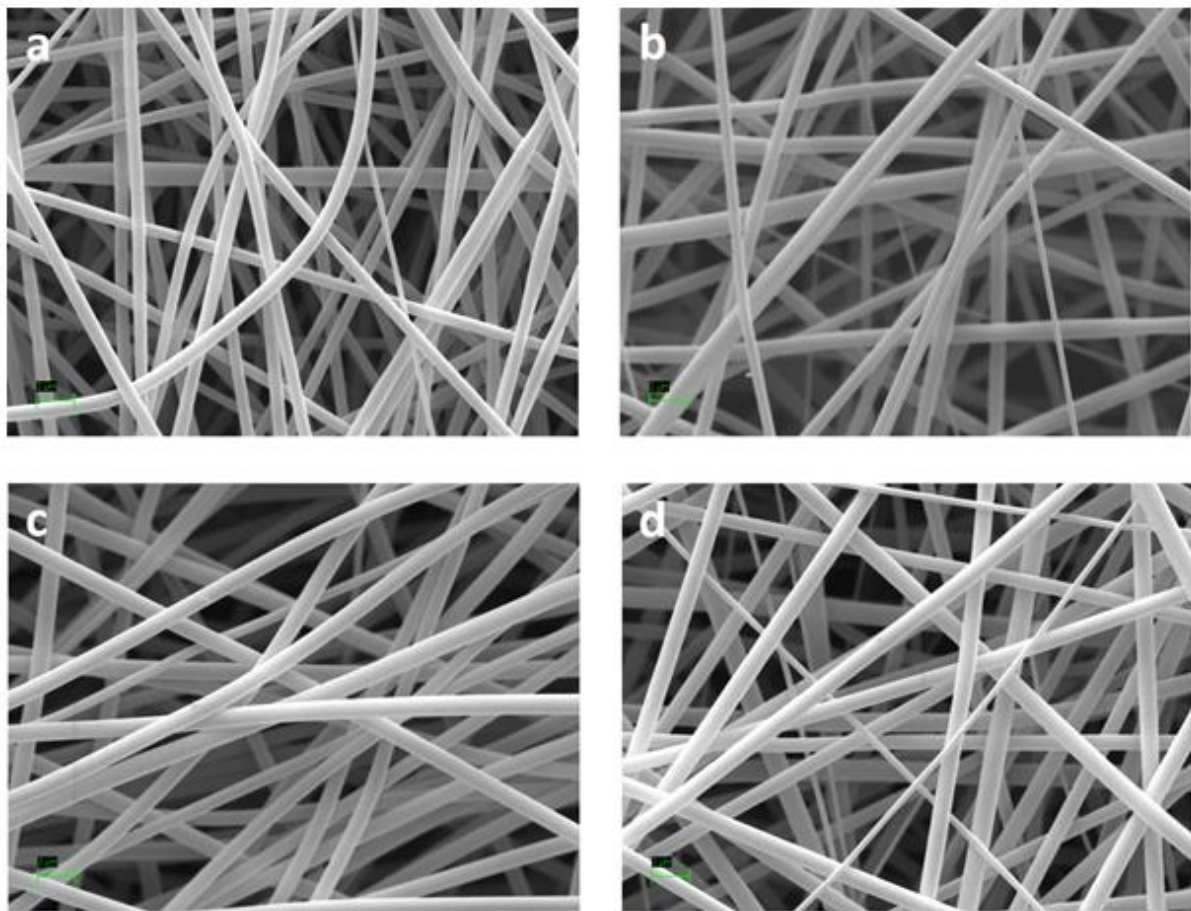


Figure 13. The morphology and structure of prepared electrospun nanofibers. Scanning electron microscopy (SEM) micrographs showing amphiphilic nanofibers of four different compositions; a) 100% polyvinylpyrrolidone (PVP) (nf1); b) 20% phosphatidylcholine (PC) and 80% PVP (nf2); c) 33.3% PC and 66.7% PVP (nf3); d) 4% chloramphenicol (CAM), 32% PC and 64% PVP (nf4). Scale bars in bottom left corners corresponds to 2 μm .

It has been shown that the morphology of nanofibers change from the straight and defect-free fibers to nanofibers with defects and beads if the applied voltage during ES is too high (Deitzel et al., 2001). The SEM micrographs (Fig. 13) confirm that amphiphilic nanofibers

with and without model drug CAM were both successfully electrospun, and that the ES processing parameters were optimal.

Casper and coworkers showed that ES in an atmosphere of less than 25 % air humidity yielded smooth polystyrene nanofibers without any surface features, while an increase of air humidity caused an increase in the number of pores on the surface (Casper et al., 2004). The nanofibers in this current study were electrospun in an atmosphere of 18-20% air humidity and showed no visible pores on the surface.

5.1.3 Diameter measurement of nanofibers

One of the most important characteristic of nanofibers is their mean diameter. This parameter enables understanding of the quality and performance of these nanofibers. Since in the present project, different nanofiber compositions were used, it was of interest to compare these nanofibers and their morphology. The mean values of nanofiber diameter were calculated by analyzing the SEM micrographs (5.1.2), and are shown with corresponding standard deviation (SD) in Figure 14.

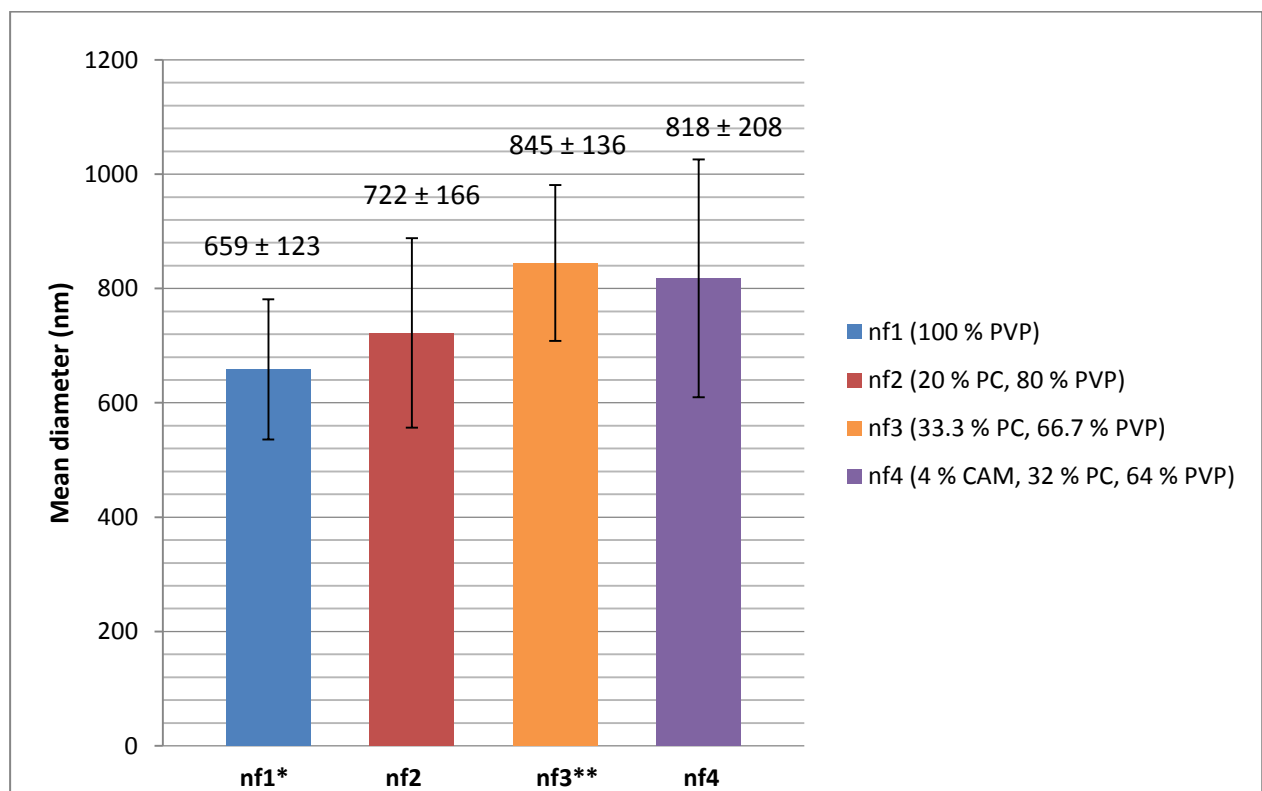


Figure 14. Diameter of four different nanofiber compositions (nf1-nf4). The values denote the mean of diameter (nm) ± SD (n=100). * nf1 shows a significant difference from nf3 (p<0.01) and nf4 (p<0.01). **nf3 shows no significant difference from nf4

Pure PVP nanofibers (nf1) from a 6% EtOH solution showed a mean diameter of 659 ± 123 nm. The diameter of the fibers increased when PC was added (nf2 and nf3), but possibly decreased when CAM was one of the components (nf4).

A statistical analysis shows that there is a significant difference between the diameters of nf1 and nf3 ($p < 0.01$) and between nf1 and nf4 ($p < 0.01$), but no significant difference between nf3 and nf4. Thus, the results imply that the presence of PC affects the morphology of the nanofibers, but the addition of 4% CAM into the formulation does not change the morphology of the nanofibers. It should be mentioned that the reproducibility of the ES was verified by using triplicate measurements. Most likely, in the present study, the processing conditions were optimized to reproduce the results.

Yu and coworkers (2011) observed the diameter of their amphiphilic nanofibers (consisting of PVP K60 and PC, with chloroform as solvent) decreasing when consisting of 20% and 33.3% PC compared to 100% PVP nanofibers. The same phenomenon was not observed in the present study. A variety of elements such as temperature, air humidity, polymer and organic solvent could have an influence on the fiber morphology (Li and Wang, 2013, Mitupatham et al., 2004). It has been shown that by varying the polymer concentration the solution viscosity may change, and the solution viscosity is directly linked to the nanofiber diameter and electrospinnability of the polymer solution (Fong et al., 1999, Hu et al., 2014). Most likely in the present study the addition of PC increased the nanofiber diameter due to increased viscosity in the solution. The morphology and diameter of electrospun nanofibers depend largely on the intrinsic properties of the solution, type of polymer, conformation of polymer chain, viscosity, elasticity and electric conductivity.

5.2 Characterization of fiber-hydrated and film-hydrated liposomes

5.2.1 Optical microscopy of fiber-HL

Due to a novelty of the liposome preparation method, the liposome self-assembly from electrospun nanofibers was controlled *in situ* and imaged using optical microscopy. Two fiber-HL dispersions without drug (pure polymer and PC) were prepared and investigated under the microscope. The images are shown in Figure 15. The images display how the liposomes have a tendency to agglomerate in very concentrated dispersions.

The optical microscopy has its limitations, and can only magnify by X50. The liposomes which can be seen in this type of microscope are only the very largest vesicles. In more concentrated dispersions where the liposomes agglomerate (Fig. 15b-c), it is easier to observe these nanostructures. However, the results showed that this preparation method (fiber-hydration) can be used for liposome preparation and was used further for preparing the drug-loaded liposome formulations.

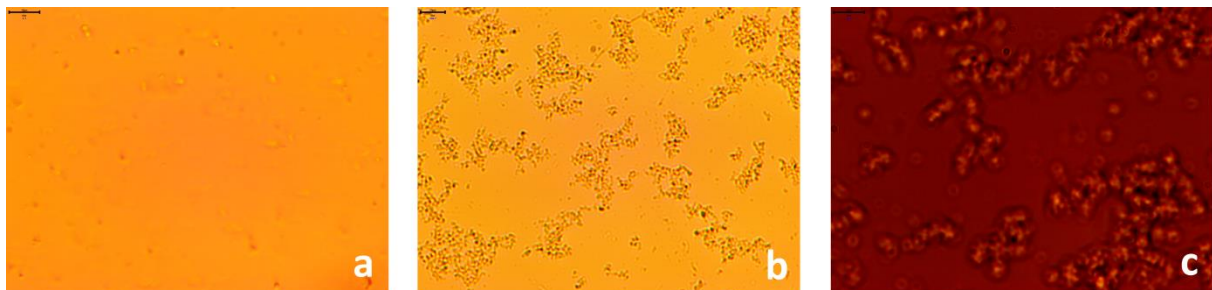


Figure 15. Optical microscopy images showing the liposomes self-assembled from hydrated nanofibers; a) 50 mg nf2 + 5 ml H₂O (X50), yellow filter, scale bar corresponds to 10 μm; b) 100 mg nf2 + 1 ml H₂O + vacuum for 10 min (X20), yellow filter, scale bar corresponds to 20 μm; c) 100 mg nf2 + 1 ml H₂O + vacuum for 10 min (X50), red filter, scale bar corresponds to 10 μm.

5.2.2 Fluorescence microscopy of fiber-HL and film-HL

Different analytical and technological methods can be used to characterize the liposomes (Ruozi et al., 2011). Among others, well-known microscopic techniques have been used and their applicability in investigating the morphology of the liposome proven. Fluorescence microscopy images of fiber-HL1-2 and film-HL1-2 (Table 2) are shown in Figure 16. It is possible to detect spherical particles in both the images of the fiber-HL and the film-HL. It

can be seen that most likely the polymer itself present in samples a-b has picked up some of the rhodamine coloring. There is a lot of noise in these images and this makes it difficult to interpret the results using this analyzing method. It is hypothesized that this experiment could be used together with an ultracentrifugation step during the preparation of the rhodamine-labelled samples to remove the polymer and any other possible disturbance from the samples during the analyses of the liposomes. It has been reported previously that the sample preparation is a critical step during the characterization of nanosized liposomes, however the CLSM together with labelling has shown good potential (Ruozi et al., 2011).

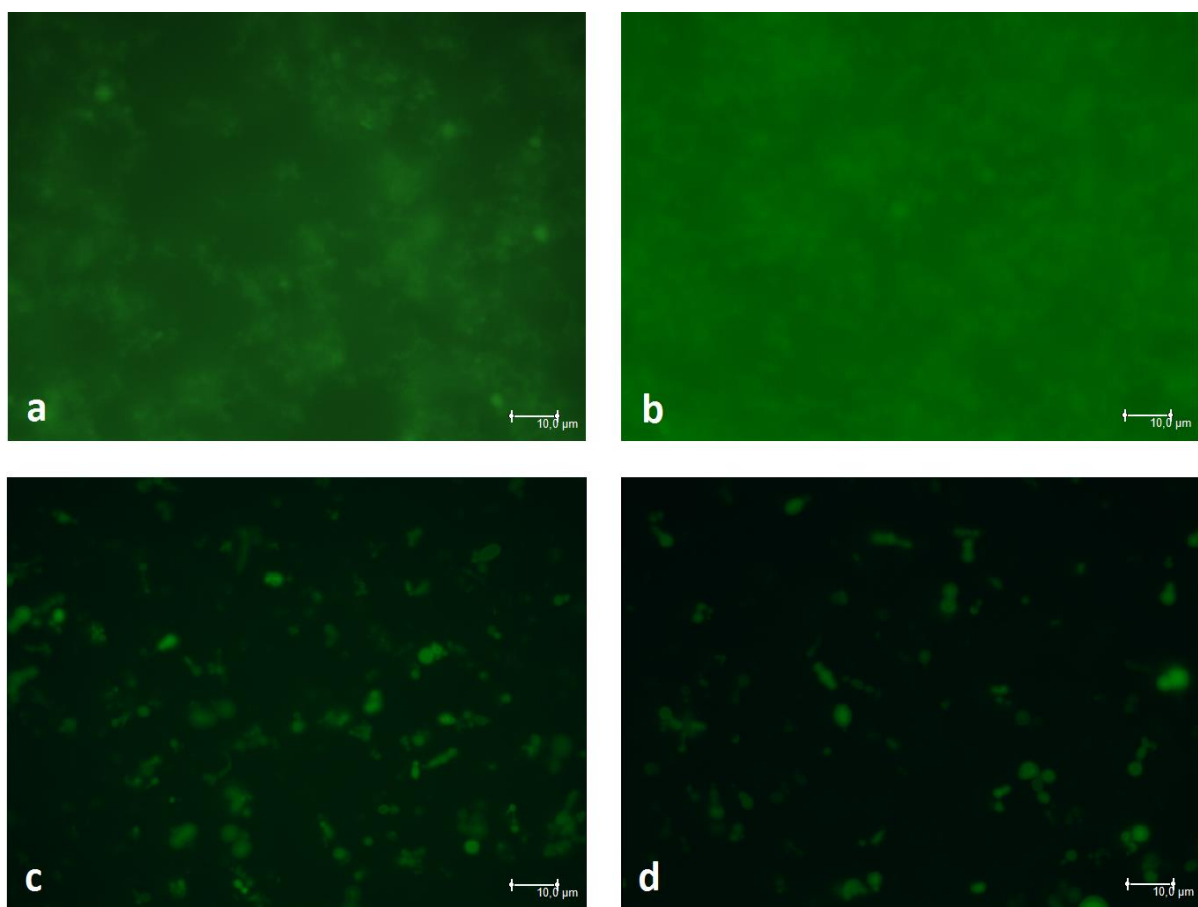


Figure 16. Fluorescence microscopy images of rhodamine-labelled liposomes; a) fiber-HL1; b) fiber-HL2; c) film-HL1; d) film-HL2. All images are magnified X100. Scale bars in bottom right corner corresponds to 10μm.

5.2.3 Particle size analysis

The morphology of the liposomes was investigated using microscopy, but the size and size distribution of the liposomes were further investigated using PCS. To investigate the diameter of the liposomes, four dispersions (film-HL1-2 and fiber-HL1-2) which included three parallels each (12 samples in total) were analyzed using PCS. Only the formulations that included drug were tested. As an example, PCS analysis graphs with lower CAM concentration are shown both for fiber- and film-HL (Fig. 17 and Fig. 18, respectfully).

Control measurements were performed with only pure polymer and PC consisting (without drug) liposomes (data not shown).

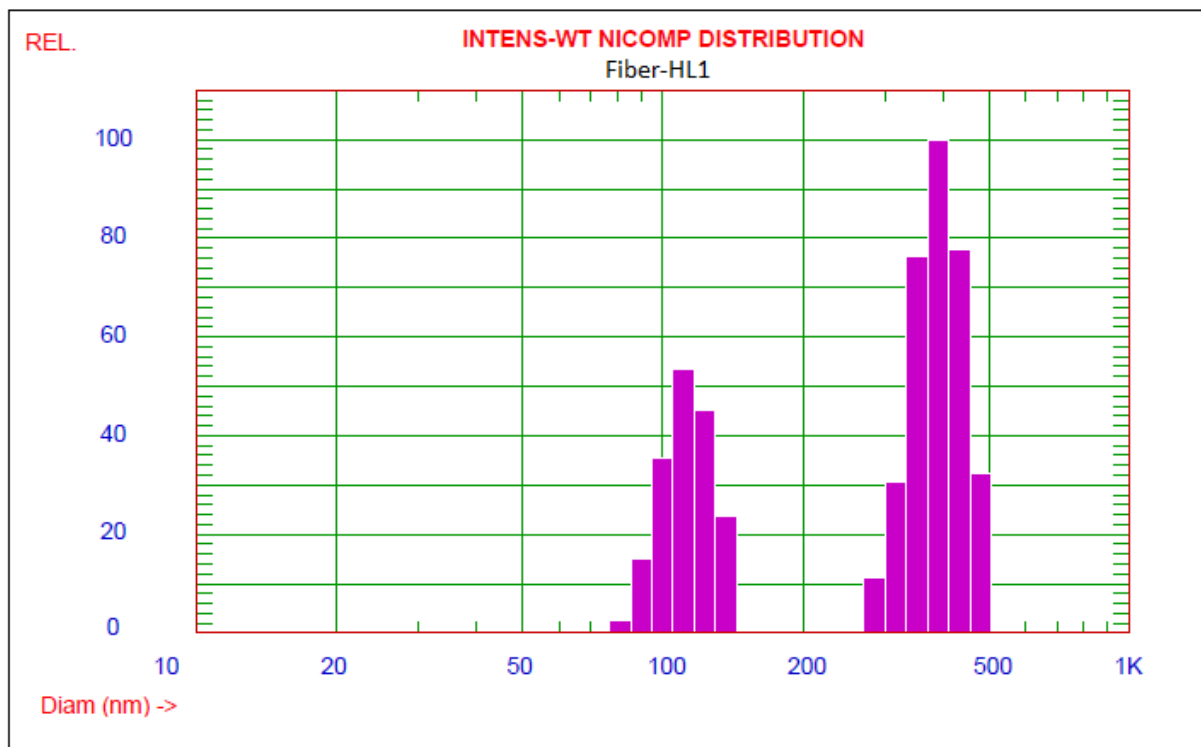


Figure 17. Example of a PCS measurement for fiber-HL1. The graph shows that two populations of different vesicle size were detected, both of them within the range of the instrument.

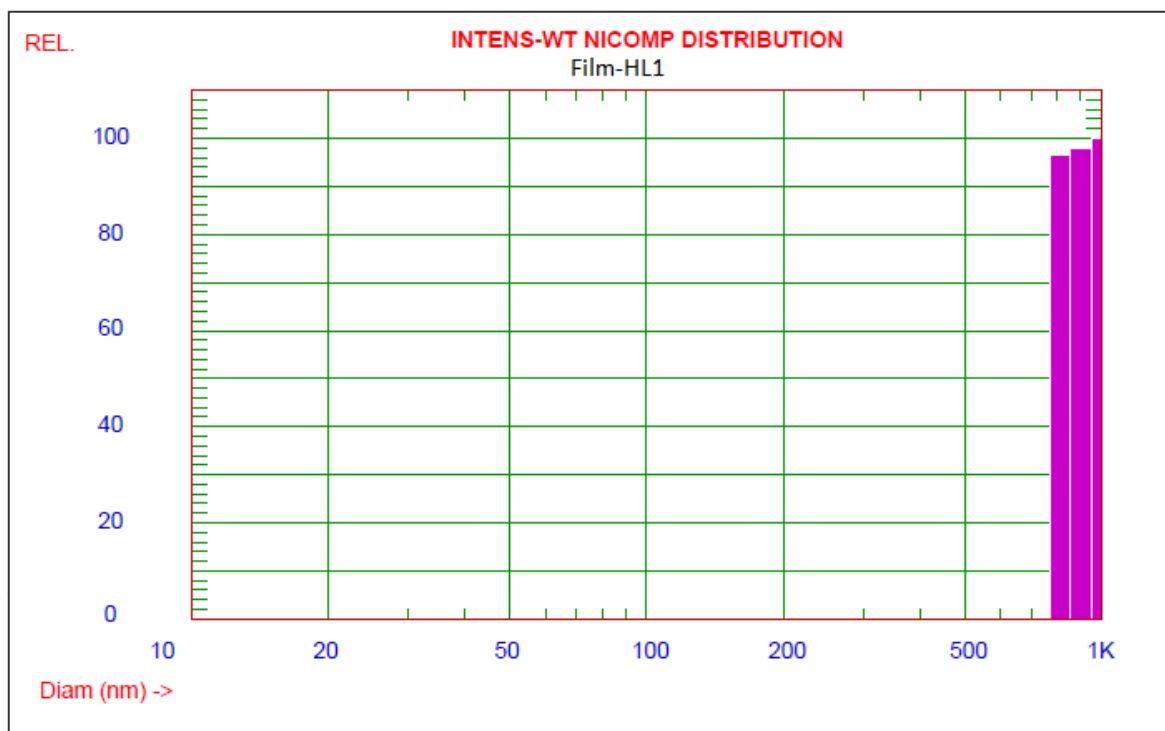


Figure 18. Example of a PCS measurement for film-HL1. The graph shows that the diameter size of the vesicles in the liposome dispersion is outside the range of the instrument.

The PCS analysis showed that the preparation technique through hydration of nf4 (fiber-HL1) yielded a polydisperse distribution with two populations. One representative measurement was chosen to represent the batch (Fig. 19 and Table 4). The largest percentage of the vesicles in this sample (61.5%) had a mean diameter of 671.3 ± 91.0 while a smaller percentage (38.5%) of the liposomes had the diameter 110.3 ± 12.7 , as shown in Table 4.

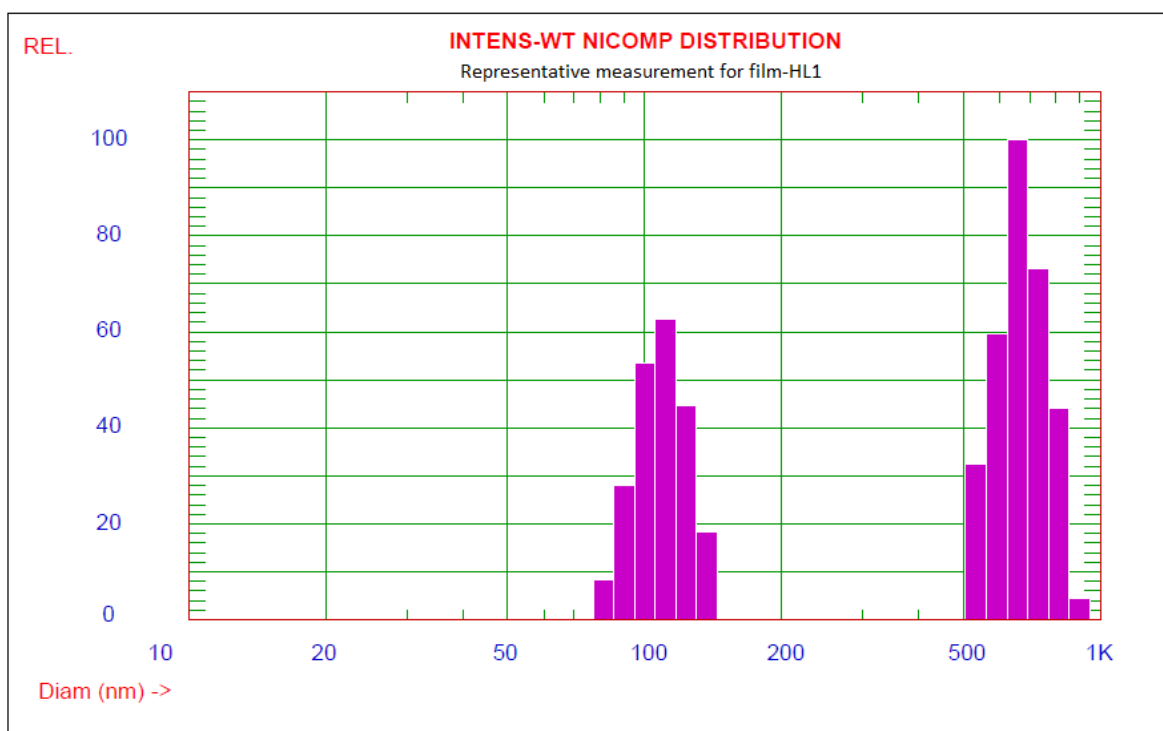


Figure 19. The measurement which was chosen to represent the measurements for fiber-HL1. The sample was polydispersed, and the graph shows the two populations detected.

Table 4: Vesicle diameter for the three liposome series which were polydisperse

Sample	Peak 1 (nm)	% of dispersion	Peak 2 (nm)	% of dispersion	Comment
Film-HL1	-	-	-	-	Out of range*
Film-HL2	-	-	-	-	Out of range*
Fiber-HL1	110.3 ± 12.7	38.5	671.3 ± 91.0	61.5	-

*The value was out of the instruments analysis range

The hydrated nf5 (film-HL2) yielded a monodisperse population, and is therefore not included in Table 4. A Gaussian distribution showed an average of the *mean diameter of* 110.5 ± 5.3 (mean ± SD, n=3). One of the measurements is shown in Figure 20.

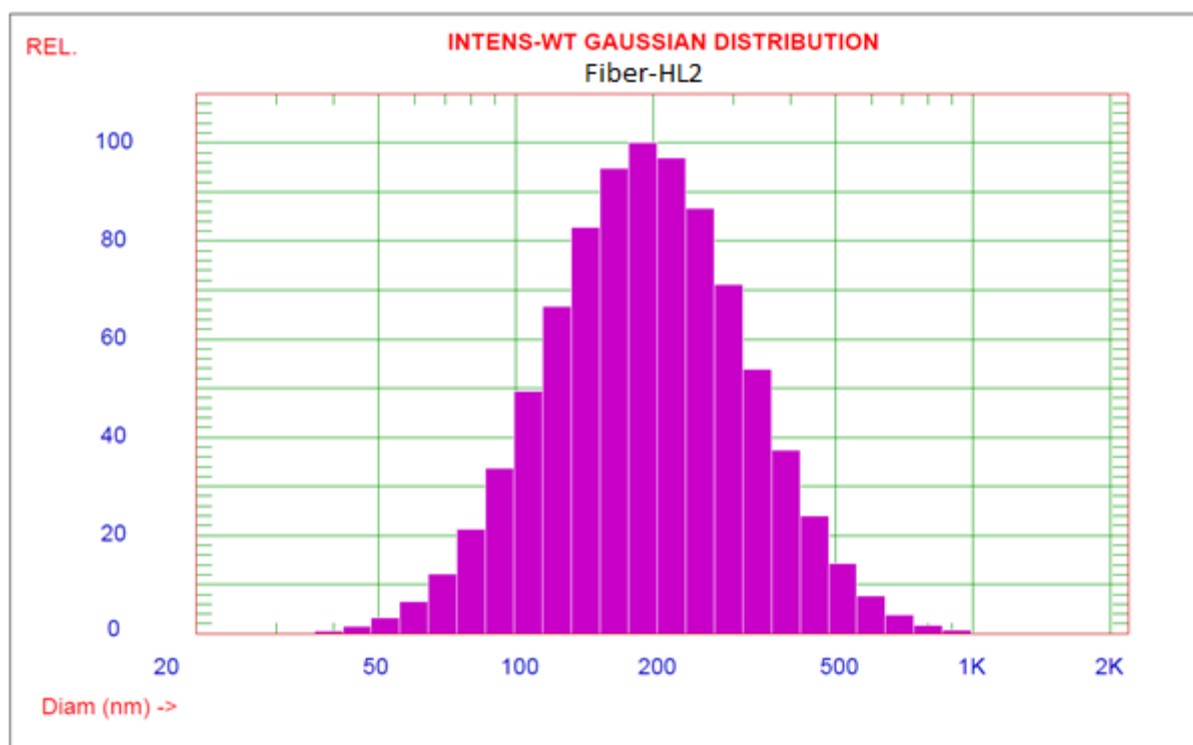


Figure 20. One of the measurements for fiber-HL2. The graph shows a monodisperse distribution of vesicle sizes in the dispersion.

The measurements from both of the film-HL were all outside the range of the PCS instrument (Table 4). It is reasonable to assume that these vesicles' diameters are too large to be measured (>800 nm) since it is common that self-assembled liposomes from a hydrated thin-lipid film yields a population of MLV's (2.1.5.1 and Fig. 4).

Film-HL1-2 showed a polydisperse distribution (Table 4). Since sizes of both of the film-HL's were outside the range of the instrument, it is not certain if they actually were polydisperse or not.

If a few larger particles are present in a sample for PCS analysis, the instrument tends to overlook very small vesicles below 30 nm (Ingebrigtsen and Brandl, 2002). This will often be the case for self-assembled liposome dispersions which has not been size reduced. In other words, there might be some very small vesicles present in the samples which have been neglected by the analyzing instrument. In the present study, it was of interest to investigate the vesicle size for the self-assembled liposomes prior to a prospective size reduction to see

how the liposomes naturally formed. For future experiments, it would be interesting to see if the fiber-HL's respond to size reduction in the same manner as film-HL's.

5.2.4 Drug encapsulation efficiency

The entrapment efficiency was determined to compare the encapsulation of drug in liposomes prepared by different methods. Both film-HL1-2 and fiber-HL1-2 samples were analyzed using HPLC analysis. The results are presented in Figure 21. A statistical analysis shows that there is a significant difference between the encapsulation efficiency of film-HL1 and fiber-HL1 ($p < 0.01$) and between film-HL2 and fiber-HL2 ($p = 0.01$).

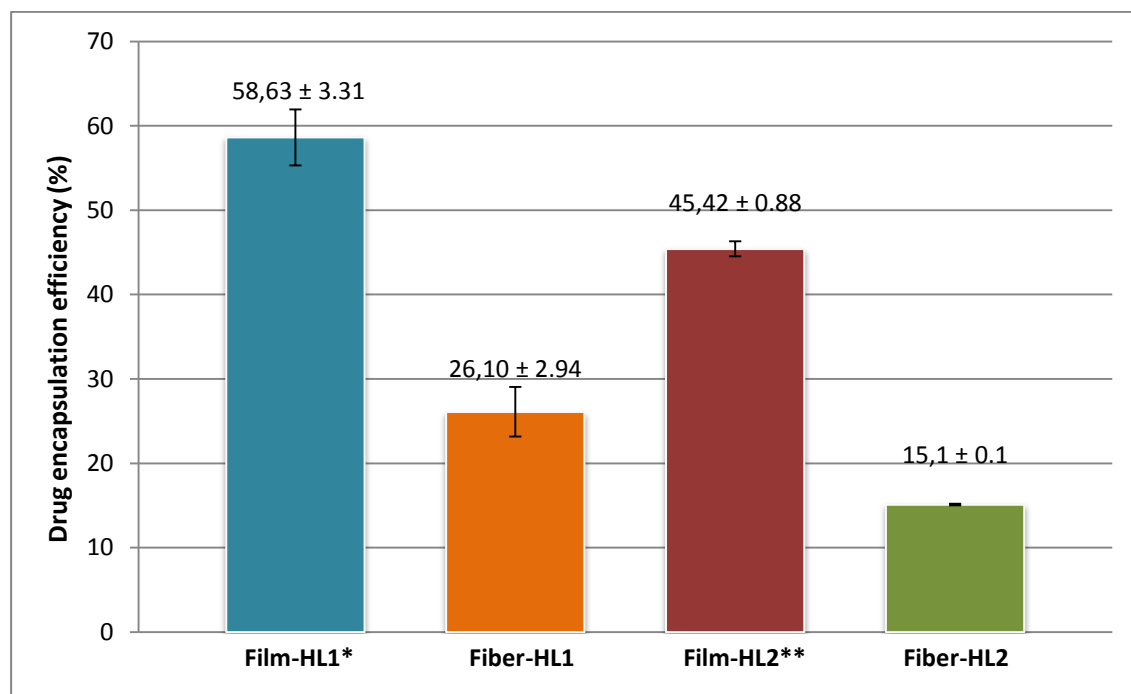


Figure 21. Drug encapsulation efficiency of film-HL1-2 and fiber-HL1-2. The values denote the mean of drug encapsulation efficiency (%) \pm SD ($n=2-3$). *Film-HL1 shows a significant difference from fiber-HL1 ($p < 0.01$). **Film-HL2 shows a significant difference from fiber-HL2 ($p = 0.01$).

The fiber-HL1, containing the lower amount of CAM, had a drug encapsulation efficiency of 26.1 ± 2.9 , while the fiber-HL2, containing the higher amount of CAM, had a drug encapsulation efficiency of 15.1 ± 0.1 (Fig. 21). Even though the drug encapsulation efficiency was higher for the fiber-HL1 dispersion, a greater amount of CAM (mg) was detected in the pellet of the centrifuged samples (2.9 mg compared to 1.0 mg) (Table 5). In conclusion, a higher percentage of CAM is encapsulated in the liposomes when the fiber-HL1

self-assemble, but a greater mass of CAM is encapsulated in the liposomes when fiber-HL2 self-assemble.

Table 5: Amounts of CAM in the liposome dispersions (mean value \pm SD)

Liposome dispersion	CAM in supernatant (mg)	CAM in pellet (mg)	Total (mg)	Theoretical amount of CAM (mg)	CAM (% of initial)
Film-HL1 n=2	5.4	7.6	12.9	14.3	90.2 \pm 5.9
Film-HL2 n=2	22.7	18.8	41.5	62.5	66.4 \pm 0.5
Fiber-HL1 n=3	2.9	1.0	3.8	4.0	95.8 \pm 1.7
Fiber-HL2 n=3	16.5	2.9	19.4	20.0	97.0 \pm 1.3

The values denote the mean for the mass of CAM and mean \pm SD for CAM (% of initial).

A noticeable large amount of CAM was lost during the fabrication of film-HL2 (Table 5). The HPLC results showed that only 66.4 % of the initial CAM amount was present within the liposome dispersion. This loss is most likely due to the poor water solubility of CAM. The thin-lipid film prepared for the film-HL2 dispersion contained 125 mg CAM, and the film was dispersed in 10 ml water. CAM is soluble in water 2.5 mg/ml (25°C) (The Merck Index, 2013), thus the loss of CAM can be explained with an insufficient dissolution of CAM. This could affect the drug amount which is captured inside the vesicles and this in turn could affect the drug encapsulation efficiency and the vesicle size. However, the drug encapsulation efficiency was determined using the real mass of CAM in the sample, and not the theoretical amount.

This CAM solubility issue and consequent problem was not present when fabricating the fiber-HL. This is because the fibers (100 mg) contained 4mg or 20 mg of CAM, and they were hydrated with 5 ml H₂O.

It is fair to assume that a loss of CAM happened during the preparation of the film-HL for the particle size analysis (5.2.3) as well, and this could have an impact on the size of the vesicles assembled. This would not be detectable on the results in this study due to the fact that all of the particle size measurements for the film-HL were outside the range of the measuring instrument. The same phenomenon was observed for the film-HL dispersions.

The PCS analysis suggests that the mean diameter of the fiber-HL vesicles is significantly smaller than the film-HL vesicles, and this can explain why the drug encapsulation efficiency is significantly smaller for the fiber-HL. It is natural to assume that the vesicles are not able to capture as much drug because of their reduced size, and thus reduced carrying space for a drug. Previously it has been shown that the liposome encapsulation efficiency is greatly dependent on the vesicle size and the lamellarity of the liposomes (Betageri and Parsons, 1992, Vemuri and Rhodes, 1995). Berger et al. (2001) fabricated drug-loaded liposomes of different sizes using filter-extruders, and found that the encapsulation efficiency correlated quite well with the sizes of the liposomes (Berger et al., 2001).

5.3 Comparison of fiber-HL and film-HL preparation methods and the prepared liposomes

Physicochemical analysis data generated in the present study allowed comparison of the drug-loaded liposomes prepared by conventional film-hydration method with drug-loaded liposomes prepared by nanofiber hydrated method. Liposomes prepared by different methods were compared for their entrapment efficiency, vesicle size and polydispersity. In both methods, the liposomes self-assembled during hydration phase, but the results confirmed that the liposome formation mechanism differs. Regardless of the preparation method used, both methods allowed preparation of drug-loaded liposomes. However, the mean diameters of liposomes differed considerably as well as the entrapment efficiency. Both the microscopy and PSC showed that formed liposomes were statistically different from each other. The fiber-hydrated liposomes showed a polydispersity with the lower CAM concentration, and monodispersity with a higher CAM concentration. Further investigation needs to be done to determine how the CAM concentration and other nanofiber compositions influence the number of liposome populations in the dispersion.

The shelf life of the liposomes is one of the biggest challenges when it comes to liposome applications. A pharmaceutical delivery system has to be physically stable, and not interfere with the chemical properties of the drug, otherwise it will not work in a proper or predictable manner (Vemuri and Rhodes, 1995). Yu and coworkers suggested that the pre-product of the fiber-hydrated liposomes, the solid amphiphilic nanofibers, could be used to store «frozen» liposomes (Yu et al., 2011). This present study has shown that *drug-loaded* liposomes also can be prepared using this novel method. It opens the opportunity to store amphiphilic nanofibers containing drug, and hydrate the nanofibers right before use to self-assemble the liposomes. Most likely a smaller liposome vesicle size could be achieved without any additional extrusion step. How the different nanofiber compositions affect the assembling and size of the liposomes and the stability of both nanofibers and liposomes should be investigated further to understand how to best exploit this drug-delivery system.

6 Conclusions

It was possible to prepare the drug-loaded liposomes *in situ* using the hydration of phospholipids deposited on the electrospun amphiphilic nanofibers. Electrospinning (ES) was found to be a suitable method for preparing amphiphilic nanofibers. Bead-free nanofibers with a smooth surface and a diameter ranging from 659 to 818 nm were successfully produced by means of ES. Microscopic techniques as well as photon correlation spectroscopy allowed performing liposome physicochemical characterization. High-performance liquid chromatography (HPLC) results showed drug encapsulation efficiency. The self-assembled liposomes made from hydrated amphiphilic nanofibers showed a lower drug encapsulation efficiency compared to film-hydrated liposomes. The liposome dispersion made from hydrated amphiphilic nanofibers showed an overall smaller vesicle size, and monodispersity when the drug amount was increased. The present nanotechnology self-assembly approach opens up new options for the fabrication, stabilization and delivery of drug-loaded liposomes.

7 Perspectives

Short-term perspectives:

- Short-term and long-term stability studies of both the nanofibers and liposomes to determine the optimal storage conditions. Could the polymer have an influence on the liposome stability due to increased viscosity of the dispersion?
- To test and compare the lamellarity of liposomes. Testing of other concentrations of drug and phospholipid. What is the ideal composition of the nanofibers prior to hydrating? How does the drug affect the assembly and characteristics such as vesicle size and polydispersity of the liposome product?

Long-term perspectives:

- Drug release studies for nanofibers prior to hydrating and fiber-hydrated liposomes
- Incorporating both film-hydrated and fiber-hydrated liposomes into nanofibers using a coaxial electrospinning (ES) set-up and eventually comparing the relevant properties.
- To map out the drug-release profile for nanofibers. How will the drug-release profile for the drug-loaded amphiphilic nanofibers differ from drug-loaded nanofibers without phospholipids?
- Evaluating applications of the drug-loaded amphiphilic nanofibers. Could nanofibers be used as an intermediate product for liposomes which are hydrated prior to use? Could the nanofibers be used as wound dressing?

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9 Appendices

9.1 Conference abstract

The enclosed abstract was granted the “Best Abstract Award” in the Fourth Annual Nordforsk Meeting on Future Performance Testing of Pharmaceutical (FPTP). The work was presented as an oral presentation in the conference (Copenhagen, January 2015). The present abstract with some modification and updated results was also accepted for publication in the 6th BBBB Conference on Pharmaceutical Sciences (Helsinki, September 2015).

Development of electrospun amphiphilic nanofibers for the *in situ* preparation and delivery of drug-loaded liposomes

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PURPOSE

The objective of the present study was to develop and evaluate the electrospun amphiphilic nanofibers intended for templating and preparing drug-loaded liposomes. The present approach exploits the hydration of phospholipids deposited on electrospun nanofibers for the formation of liposomes.

INTRODUCTION

The physical stability is the primary challenge in formulating liposomes for drug delivery applications. A novel strategy for using electrospun composite nanofibers as templates in fabricating liposomes was recently introduced by Yu et al. (1). To date, this strategy has not been applied for fabricating drug-loaded liposomes.

METHODS

The amphiphilic nanofibers were prepared using a ESR200RD robotized electrospinning system (NanoNC, Korea). A conventional film hydration method was used as a reference method for preparing liposomes. Polyvinylpyrrolidone (PVP 90) and soybean phosphatidyl choline were used at different concentrations as a filament-forming matrix. The liposome formation were monitored *in situ* by using optical microscopy. The geometric properties and surface topography of nanofibers were characterized by scanning electron microscopy (SEM). The physical solid-state analyses were made by Raman spectroscopy, X-ray powder diffraction (XPRD) and differential scanning calorimetry (DSC).

RESULTS

Electrospinning of phospholipids (soybean phosphatidyl choline) with PVP using ethanol as a solvent system was found to be successful in fabricating amphiphilic composite nanofibers. According to the optical microscopy results, the immediate hydration of phospholipids deposited on the amphiphilic nanofibers occurred within few seconds resulting in the formation of liposomes in water. The formation of liposomes were studied in both concentrated and diluted solutions, and the liposomes appeared to aggregate more readily in the concentrated solutions than in the diluted solutions.

CONCLUSION

It is possible to prepare liposomes *in situ* using the hydration of phospholipids deposited on electrospun amphiphilic nanofibers. The present nanotechnology self-assembly approach opens up new alternatives for the fabrication, stabilization and delivery of liposomes.

ACKNOWLEDGEMENTS

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9.2 SEM micrographs

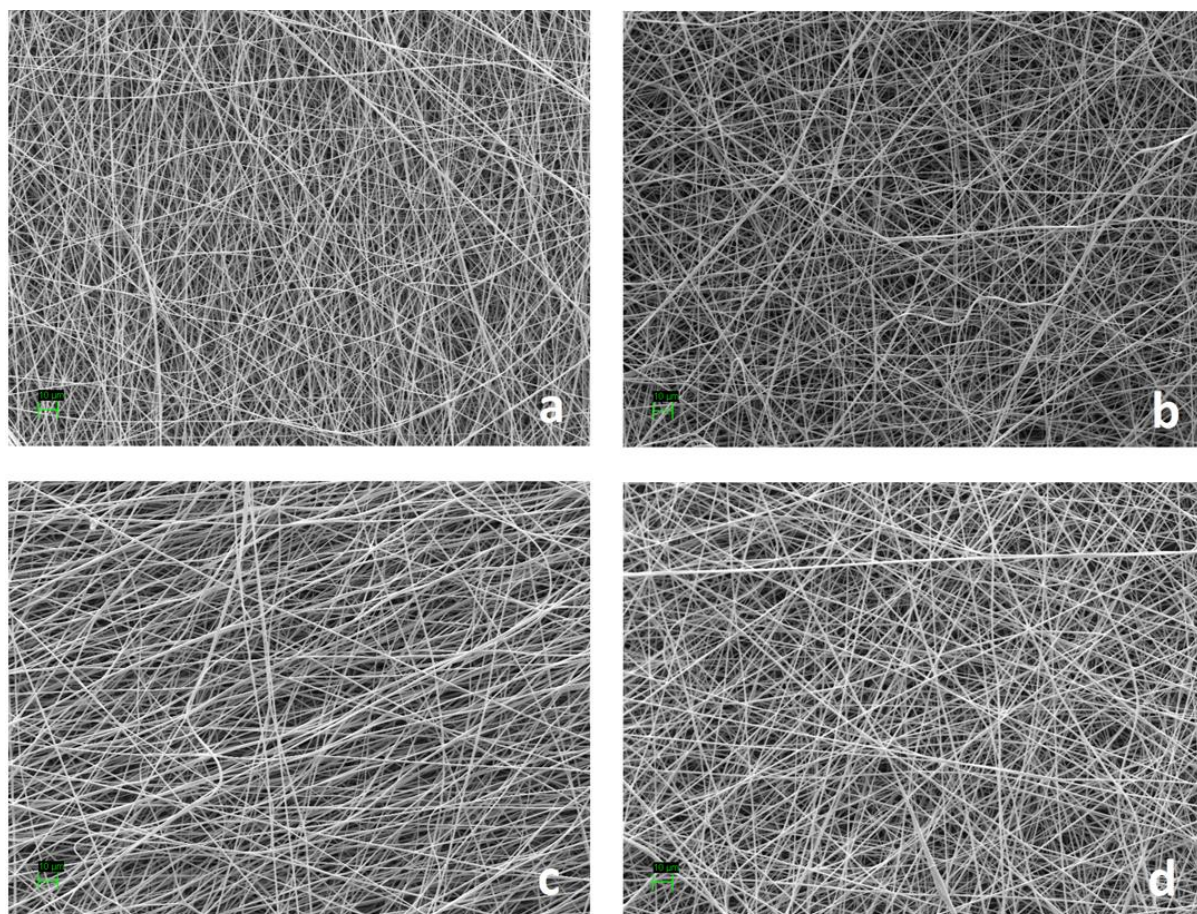


Figure 22: The morphology and structure of prepared electrospun nanofibers. Scanning electron microscopy (SEM) micrographs showing amphiphilic nanofibers of four different compositions; a) 100% polyvinylpyrrolidone (PVP) (nf1); b) 20% phosphatidylcholine (PC) and 80% PVP (nf2); c) 33.3% PC and 66.7% PVP (nf3); d) 4% chloramphenicol (CAM), 32% PC and 64% PVP (nf4). Scale bars in bottom left corners corresponds to 10 μm .