



UIT

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Department of Pharmacy  
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## Can nanomedicine improve the semen quality?

*The potentials of liposomal curcumin.*

—  
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## Sammendrag

**INTRODUKSJON:** På verdensbasis rammes 10-15 % av befolkningen av infertilitet, og 50 % av parene som søker om assistert befruktning gjør dette på grunn av nedsatt sædkvalitet. Sædkvaliteten er nært knyttet til oksidativt stress (OS). På grunn av spermienes store energibehov og svake antioksidant mekanismer har spermene vanskeligheter med å forsvare seg mot OS produsert både eksogent og endogent. Vitamin C og/eller vitamin E er antioksidanter med evne til å redusere OS, og kliniske utprøvningsviser forbedringspotensiale. Gurkemeie er en ekstrem potent, lipofil antioksidant som har vist potensiale i behandling av flere sykdommer hos mennesker, men dårlig biotilgjengelighet har så langt begrenset gurkemeies kliniske bruk. For å bedre biotilgjengeligheten til gurkemeie er nanoteknologi tatt i bruk. Det er derfor interessant å undersøke om nanoteknologi-baserte liposomer kan øke den antioksidative effekten av gurkemeie. Dette for å oppnå forbedret bevegelighet og levedyktighet av spermier *in vitro*.

**FORMÅL:** Målet med denne oppgaven var å demonstrere antioksidativ aktivitet av gurkemeie, samt å undersøke om en liposom-formulering med gurkemeie kan øke denne antioksidative aktiviteten og forhåpentligvis forbedre bevegelighet og levetiden til spermene *in vitro*.

**METODE:** Liposomer med gurkemeie ble fremstilt ved bruk av film-metoden og filterekstrudering. Den antioksidative effekten av gurkemeie ble testet med ABTS- og DPPH-redoksindikatorer, og sammenlignet med vitamin C og vitamin E. Effekten av tomme liposomer, liposomer med gurkemeie, fritt gurkemeie, vitamin C og vitamin E ble bestemt ved å observere bevegeligheten og levedyktigheten til spermier *in vitro*.

**RESULTATER:** Liposomene med gurkemeie hadde en størrelse på  $147,82 \pm 17,3$  nm, og av tilsatt gurkemeie var  $88,84 \pm 2,31$  % inkorporert i liposomene.  $EC_{50}$  for gurkemeie ble funnet til å være  $5,82 \mu\text{M}$  i ABTS og  $9,13 \mu\text{M}$  i DPPH. Antioksidantene som fritt gurkemeie, vitamin C og vitamin E opprettholdt bevegeligheten og levedyktigheten i opptil 24 timer. Lipid i konsentrasjoner over  $0.5 \mu\text{g/ml}$  viste toksisk effekt på spermene.

**KONKLUSJON:** Liposomene med curcumin var uniforme i størrelse og hadde en høy inkorporeringsevne. Det ble bekreftet at gurkemeie hadde antioksidative egenskaper, og studien viser at liposomale lipider overraskende hadde negativ effekt på levedyktigheten til spermene. Flere studier med flere paralleller må midlertidig utføres før dette kan bekreftes.

**Nøkkelord:** gurkemeie, liposomer, antioksidant, sperm, bevegelighet, levedyktighet



## Abstract

**INTRODUCTION:** Worldwide, 10-15 % couples are infertile, and 50 % of the couples seeking assisted reproductive techniques do so because of defective sperm quality. The semen quality can be directly linked to oxidative stress (OS). Due to high metabolic rates and weak anti-oxidant systems, sperm cells are unable to defend against OS produced exogenously and endogenously. Vitamin C and/or vitamin E are anti-oxidants showing promising outcome to reduce OS, but the clinical application has not been fully achieved. Curcumin is an extremely potent lipid-soluble anti-oxidant showing potential in therapy for different human disorders, but its poor bioavailability has limited its wider use. Nanotechnology has been used for the purpose of improving the bioavailability of curcumin. It would be interesting to apply nanotechnology based liposomal delivery of curcumin, a noted anti-oxidant for the enhancement of motility and viability of human sperm cells *in vitro*.

**OBJECTIVES:** With this study, the aim was to prove anti-oxidative activities of curcumin. We also studied if liposomal formulation of curcumin could maximize the anti-oxidative activities and hopefully enhance motility and viability of sperm cells *in vitro*.

**METHODS:** Curcumin loaded liposomes were made by the film method followed by extrusion. Anti-oxidative activities of free curcumin were tested with ABTS and DPPH assay methods and compared with vitamin C and vitamin E. The effect of empty liposomes, liposomal curcumin, free curcumin, vitamin C and vitamin E was determined by observing the motility and viability of sperm cells *in vitro*.

**RESULTS:** Liposomes with curcumin had an average size of  $147.82 \pm 17.3$  nm and entrapment efficiency of  $88.84 \pm 2.3$  %. The  $EC_{50}$  for curcumin was  $5.82 \mu\text{M}$  in ABTS and  $EC_{50}$   $9.13 \mu\text{M}$  in DPPH assay, respectively. Anti-oxidants such as curcumin, vitamin C and vitamin E maintained the motility and viability up to 24 hours, however lipid showed toxicity to sperm cells at  $0.5 \mu\text{g/ml}$  or higher concentrations.

**CONCLUSION:** The liposomes containing curcumin were uniformly distributed with high entrapment efficiency. We confirmed anti-oxidative activity of curcumin, however, rather unexpectedly that the liposomal lipid has shown negative effect on sperm viability at certain concentrations. Further studies with more replicates are needed to confirm these findings.

**Key words:** curcumin, liposomes, anti-oxidant, sperm, motility, viability



## List of abbreviations and symbols

ABTS	2,2'-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt
ABTS <sup>+</sup>	cationic ABTS in free radical form
ATP	adenosine triphosphate
DMSO	dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
DPPH <sup>•</sup>	DPPH in free radical form
EE	entrapment efficiency
GUV	giant unilamellar vesicle
LPO	lipid peroxidation
LUV	large unilamellar vesicle
MLV	multilamellar vesicle
OS	oxidative stress
PC	phosphatidylcholine
PPOS	potassium peroxydisulfate
RES	reticuloendothelial system a.k.a. macrophage system
RNS	reactive nitrogen species
ROS	reactive oxygen species
SUV	small unilamellar vesicle
$\chi^2$	chi square



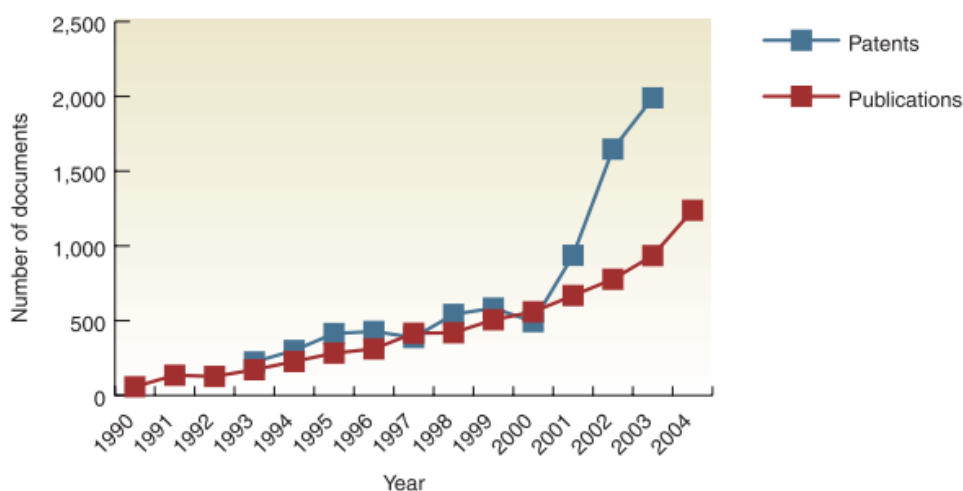


# 1. INTRODUCTION

## 1.1 Nanomedicine

**Nanomedicine** is defined by the National Institutes of Health in the USA as “the use of nanotechnology for treatment, diagnosis, monitoring and control of biological systems” (Moghimi et al., 2005). The physical definition of nanomedicine states nanostructured materials in medicine that have a size between 1 and 100 nm (Wagner et al., 2006). It should be noted that the treatment of diseases based on drug particle size is a recently developed medical field.

The publication and patent activities on nanomedicines have risen rapidly since year 2000 (*Figure 1*). This shows that nanotechnology in the medical field has gained tremendous interest because of enabling functions to existing products making them competitive. Nanopharmaceutics are favoured in biomedicine mostly because of their promising properties as **drug delivery systems** (Wilczewska et al., 2012, Naahidi et al., 2013, Barkalina et al., 2014). Drug delivery systems are developed for the improvement in bioavailability and pharmacokinetics in therapeutics. An example of this kind is a market product “Ambisome” which is a liposomal formulation based drug that showed reduced toxicity to kidney with improved fungicide function (Wagner et al., 2006). Drug delivery systems are the main area and are accounting for more than 75 % of the total sales in the field of nanomedicine (Wagner et al., 2006). **Twenty three** nanomedicines based drug delivery systems were already available commercially in 2006 (Wagner et al., 2006), and the number was rapidly increased to 71 in 2013 (Etheridge et al., 2013).

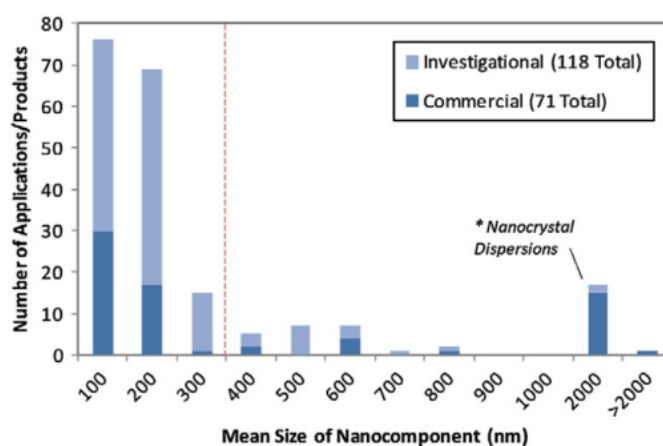


**Figure 1:** Patents and publications activities worldwide on nanomedicine to year 2005 (Wagner et al., 2006) (with permission).

Nanotechnology was first applied for cancer diagnostics and treatment by destroying unwanted cells with high precision. The improvement of nanotechnology and safety have led to expansion in use of nanotechnology in different non-cancer applications. Among one of them is reproductive medicine (Barkalina et al., 2014).

Application of nanomaterials for therapeutic purpose has been competitive to conventionally established theories because of changed chemical, physical and biological properties due to particle size (1-100 nm). Compared to other bulk solid materials, nanomaterials are more controlled of quantum mechanics than material physics (Barkalina et al., 2014).

Characteristics of nanomaterials are the particle size ranges from 1 to 1000 nm as well as the type of materials. Most of the market products as nanomaterial drugs had a particle size between 100 and 200 nm in 2013 (*Figure 2*)(Etheridge et al., 2013). Small sizes of nanoparticles have shown better integrating possibilities into cellular processes and physiological pathways without interfering with normal biological system. Nanomaterials used in the drug delivery have great potentials to carry large amounts and different types of biological cargo. Nanosystem protect drug from rapid clearance and degradation by reticuloendothelial system (RES). Surface can be modified to react with environmental factors giving responsive drug release (Wilczewska et al., 2012, Naahidi et al., 2013, Barkalina et al., 2014).



**Figure 2:** Mean size of nanocomponents in nanomedicine applications reached marked or under investigation in 2013 (Etheridge et al., 2013) (with permission).

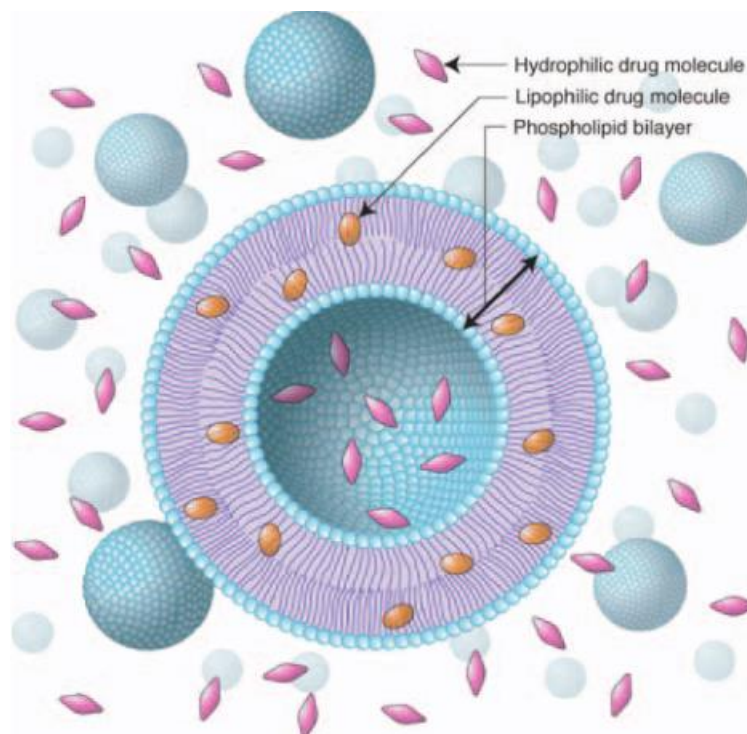
**With all these functionalities that nanotechnology can contribute with, it is interesting to see if nanotechnology can be applied for the improvement of sperm quality. In this context, it would be interesting to know whether some anti-oxidant incorporated liposomes can have an impact on the motility and viability on sperm cells.**

## 1.2 Liposomes

### 1.2.1 Liposomal characteristics

In 1965, the first description of swollen phospholipid systems was published, and these closed bilayer membrane systems are what we today call liposomes (Deamer, 2010). Liposomes are vesicles, consisting of a uni-, oligo- or multi-lamellar lipid bilayers surrounding aqueous core and are most commonly used as drug delivery systems (Wagner et al., 2006, Spuch and Navarro, 2011).

Liposomes are formed by dispersing amphiphilic compounds in aqueous media (Sharma and Sharma, 1997). When an amphiphilic compound comes in contact with water, the polar head groups interact with the water, while the tails interact with each other and parts from the aqueous medium. This forms a bilayer membrane. The advantage of this bilayer structure is that it is possible to entrap hydrophilic compounds and/or incorporate hydrophobic compounds in the lipid layer as shown in *Figure 3* (Hupfeld et al., 2006).

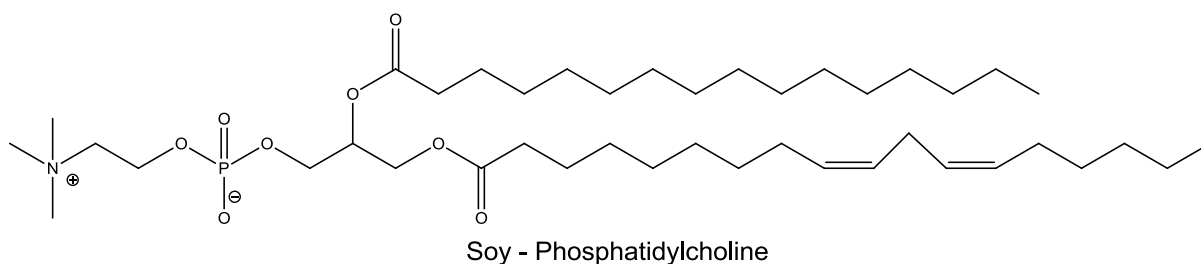


**Figure 3:** An unilamellar liposome with hydrophilic drug in aqueous compartment and lipophilic drug incorporated in phospholipid bilayer (Hupfeld et al., 2006)

## 1.2.2 Membrane component - phosphatidylcholine

One of the most used amphiphilic compounds to form the liposomal membrane are the phospholipids (New, 1990). One of the most common phospholipids used is phosphatidylcholine (PC) from egg or soy (New, 1990, Fiume, 2001, Spuch and Navarro, 2011).

The structure of PC molecule consists of two long fatty acid chains, often stearic, palmitic or oleic acids combined by a glycerol bridge (Fiume, 2001). The glycerol bridge connects the hydrophilic head which makes the amphiphilic structure to PC as shown in *Figure 4* (New, 1990). The hydrophilic head comes from a positively charged choline group and negatively charged phosphate oxygen and carbonyl oxygen atoms (Pasenkiewicz-Gierula et al., 1999). Phosphoric acid is linked to the  $\alpha$ -position of glycerol bridge in natural, but it can be attached in the  $\beta$ -position as well (Fiume, 2001).



**Figure 4:** Chemical structure of zwitterionic soy- phosphatidylcholine (PC).

PC is one of the components in lecithin (Patil et al., 2010). Soy lecithin is confirmed safe by the World Health Organization (WHO) and has been awarded Generally Recognized as Safe (GRAS) status by the US Food and Drug Administration (FDA) (World Health, 1974, Fiume, 2001). PC in concentration from 0.1 to 3.0 mM shows no significant effect on human sperm motility (Hong et al., 1986). Because of PCs safety and low costs, PC is often used to form the phospholipid membrane in liposomes (New, 1990).

### 1.2.3 Classification of liposomes

Liposomal classification after shape, size and number of bilayers:

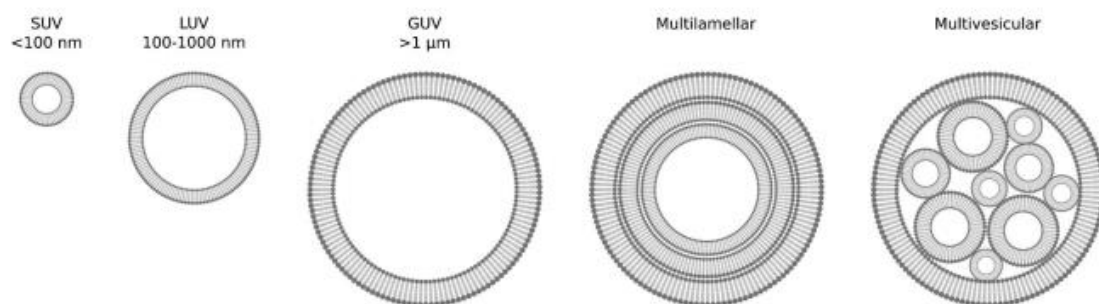
Small unilamellar vesicle (SUV): consist of one single layer of membrane with diameter between 10 and 100 nm (Sharma and Sharma, 1997, van Swaay and deMello, 2013).

Large unilamellar vesicle (LUV): consist of one single layer of membrane with diameter between 100 and 1000 nm (Sharma and Sharma, 1997, van Swaay and deMello, 2013).

Giant unilamellar vesicle (GUV): consist of one single layer of membrane with size up to 1  $\mu\text{m}$  (van Swaay and deMello, 2013).

Multilamellar vesicle (MLV): consist of many layers of membrane with diameter usually over 100 nm (Sharma and Sharma, 1997, van Swaay and deMello, 2013).

Multivesicular vesicle: consist of small vesicles inside a bigger vesicle (Sharma and Sharma, 1997, van Swaay and deMello, 2013).



**Figure 5:** Liposomes classified after their size and number of bilayers. SUV = small unilamellar vesicle, LUV = large unilamellar vesicle and GUV = giant unilamellar vesicle (van Swaay and deMello, 2013) (with permission).

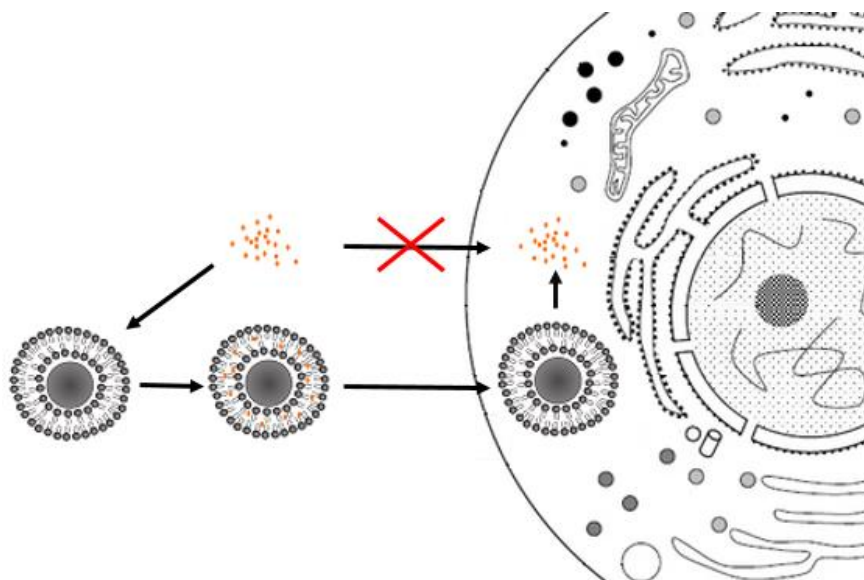
### 1.2.4 Liposomes in drug delivery

The first liposomal formulation that was approved by the US Food and Drug Administration was Doxil® in 1995, for the treatment of chemotherapy refractory acquired immune deficiency syndrome-related Kaposi's sarcoma (Chang and Yeh, 2012). The liposomal formulation gave a significantly change improved distribution and elimination, and doxorubicin was more concentrated in the tumour area (Northfelt et al., 1996).

Experiments show that some drugs incorporated or encapsulated in liposome gets less eliminated from plasma, and improved drug distribution compared to non-incorporated or non-entrapped drugs (Gregoriadis, 1973). The shape and properties of liposomes are similar to the

human cell membrane, which gives them good compatibility and low toxicity. Inserting drugs inside a vehicle make the drug more secured from degradation and sensitive tissues will be less affected (Gregoriadis, 1973, Allison, 2007). The possibilities of modifying liposomes can precisely locate and hold the drug concentration in a therapeutic level, without affecting immunological attack (Spuch and Navarro, 2011).

Conventional liposomes are the term used for liposomes that often consists of neutral and/or negatively charged phospholipid and cholesterol (Sharma and Sharma, 1997). They are taken up by endocytosis and deliver its contents to lysosomes, which are roughly explained in *Figure 6*. Liposomal size between 10-100 nm have shown optimal pharmacokinetic properties *in vivo*, while smaller are renally cleared and bigger are removed by RES in liver and spleen (Allen and Chonn, 1987, Sharma and Sharma, 1997, Wilczewska et al., 2012, Naahidi et al., 2013, Barkalina et al., 2014, Hafner et al., 2014).



**Figure 6:** A modified schematic drawing showing the cellular uptake increases when a poor absorbed drug is incorporated in liposomal formulation. Here are curcumin illustrated as orange dots (Spuch and Navarro, 2011) (with permission).

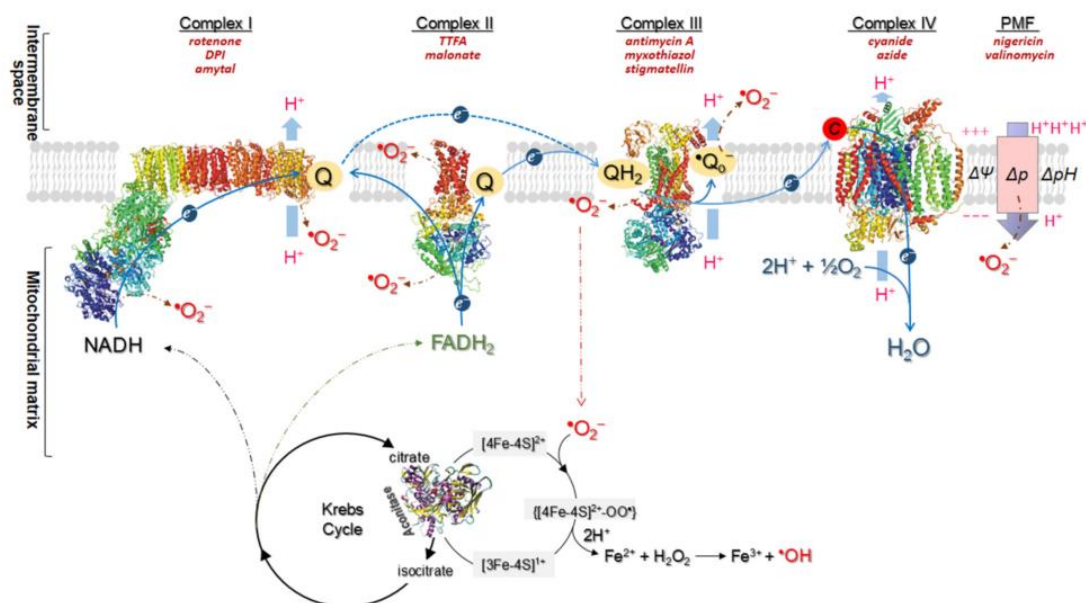
It is clear that liposomal formulation increases solubility and cellular uptake for those drugs having poor solubility and bioavailability. Liposomal formulation of curcumin was chosen because numerous liposomal formulations are done for tumour cells and red blood cells (Anand et al., 2007), but there are no articles found for sperm cells. It could be interesting to know whether a poorly soluble anti-oxidant, such as curcumin, once incorporated in liposomes can improve the sperm cells motility and viability.

### 1.3 Role of anti-oxidants in male infertility

Infertility is defined as “Women of reproductive age at risk of becoming pregnant and report unsuccessful pregnancy for more than two years” (Mascarenhas et al., 2012). Almost fifty percent of the couples seeking for assistance reproductive techniques are because of not optimal semen quality (Jensen et al., 2013). This can either be caused by low sperm count, reduced sperm motility or sperm with abnormal morphology (Krausz, 2011, Mascarenhas et al., 2012). The defective semen quality has been attributed mainly to the reactive oxygen species (ROS) and thus produced oxidative stress (OS).

ROS are produced during cellular metabolism and needed for maintaining normal cellular physiology including spermatogenesis and fertilizing ability. However, overproduction of ROS can lead to dysfunction of sperms and 30-80 % of ROS related pathology is reported in infertile patients. High ROS levels are linked with OS, giving defective sperm functions and birth defects (Sharma and Agarwal, 1996, Gilbert and Colton, 2002, Bansal and Bilaspuri, 2010, Aitken and Koppers, 2011, Agarwal et al., 2014).

The source for ROS production is in the electron transport chain in the mitochondria (Murphy, 2009, Bratic and Larsson, 2013). ROS are involved in generation of OS and oxidative damage. Mainly involved free radicals are superoxide anion ( $O_2^{\cdot-}$ ), hydroxyl (OH), nitric oxide (NO) and peroxynitrite ( $ONOO^-$ ) while the non-free radical species are hydrogen peroxide ( $H_2O_2$ ) and nitrous acid ( $HNO_2$ ) (Bansal and Bilaspuri, 2010, Basnet and Skalko-Basnet, 2011).



**Figure 7:** Presentation of the electron transport chain and mechanisms involved in free radical production (Chen and Zweier, 2014) (with permission).

A detailed mechanism of production of ROS is explained by a schematic diagram in *Figure 7*. A sudden ROS exposure affects most mitochondrial proteins that can result in ATP-synthesizing impairment (Chen and Zweier, 2014) and impairment in signalling (Droge, 2002). A long time exposure of ROS can addition to the damages as in sudden exposure also damage cellular proteins (Stadtman and Levine, 2000). This can lead to lipid peroxidation (Rubbo et al., 1994, Murphy, 2009) and DNA impairing (Turrens, 2003, Chen and Zweier, 2014).

Since sperm cells have limited capacity to defend against ROS and/or free radicals, DNA and other organelles can be damaged and affect the fertility and fetus development. It has been thought that intake of some anti-oxidants like fruits, grains and vegetables can have favourable effect against oxidative stress (Halliwell, 1994, Gilbert and Colton, 2002, Bansal and Bilaspuri, 2010, Agarwal et al., 2014). Among several anti-oxidants, vitamin C and vitamin E are two important anti-oxidants found in the natural sources and are practiced clinically for the improvement of semen quality (Kessopoulou et al., 1995, Verma and Kanwar, 1998, Agarwal et al., 2003, Lanzafame et al., 2009, Bansal and Bilaspuri, 2010).

On the other hand, curcumin is a well-known anti-oxidant that has been gaining a lot of interest markedly the past two decades because of its potent anti-oxidative and anti-inflammatory properties in the preclinical tests. In spite of its high potential as a therapeutic drug, its clinical application for treatment of human diseases is limited because of low solubility and poor bioavailability (Aitken, 1999, Bansal and Bilaspuri, 2010, Gupta et al., 2012, Chen et al., 2013, Walczak–Jedrzejska et al., 2013, Agarwal et al., 2014).

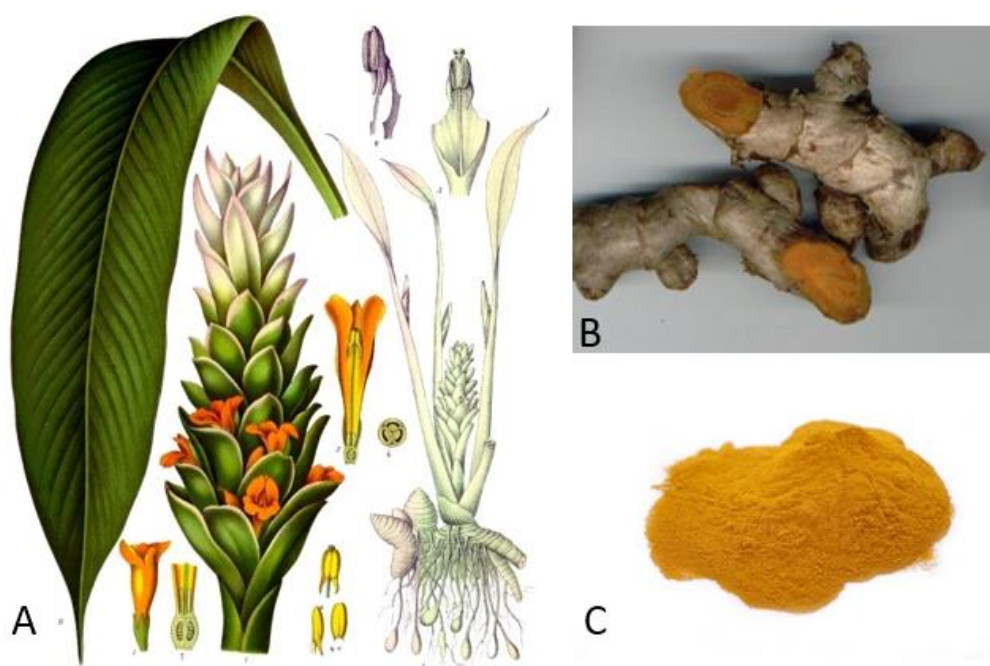
With this in mind, the purpose with this study is to see whether anti-oxidative potentials of curcumin can be utilized to reduce oxidative stress in sperm cells, and effect can be maximized by using nanotechnology as the liposomal formulation. Hopefully, it could be the tool in the assisted reproductive technology (ART) especially for the improvement of semen quality.



## 1.4 Curcumin

### 1.4.1 Origin of curcumin

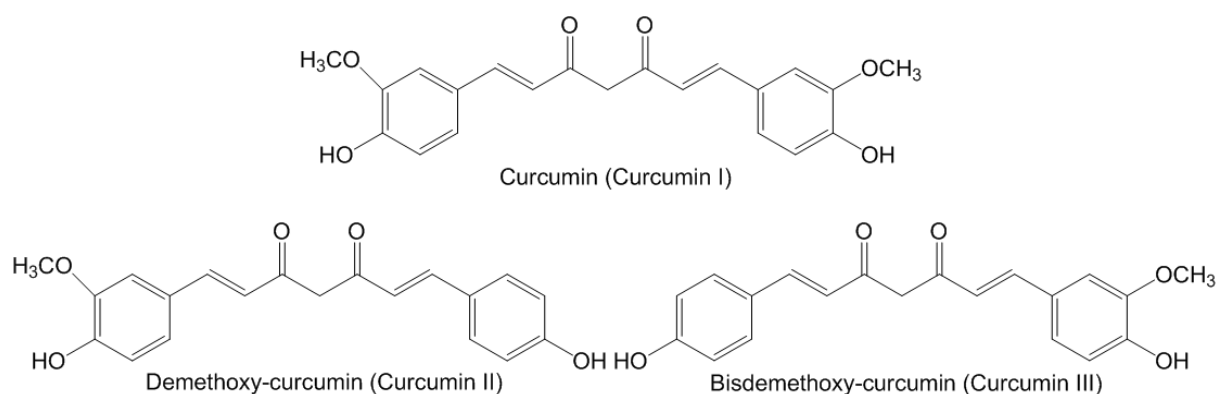
Curcumin, also known as diferuloylmethane, is a polyphenol and mainly derived from dried rhizomes from the plant *Curcuma longa* Linn, also known as “Turmeric” (Sharma et al., 2005, Goel et al., 2008, Padhye et al., 2010, Basnet and Skalko-Basnet, 2011). Turmeric has been used for treatment purposes to different inflammation, metabolism, respiratory and skin diseases in Ayurvedic medicine, which is one of the world’s oldest medical systems (Sharma et al., 2005, Goel et al., 2008, Padhye et al., 2010, Mahajan, 2011)



**Figure 8:** A: The plant *Curcuma longa* Linn, B: Rhizome of the plant and C: Turmeric powder. Pictures are taken from Wikipedia (A and C) and B are published with permission (Basnet and Skalko-Basnet, 2011).

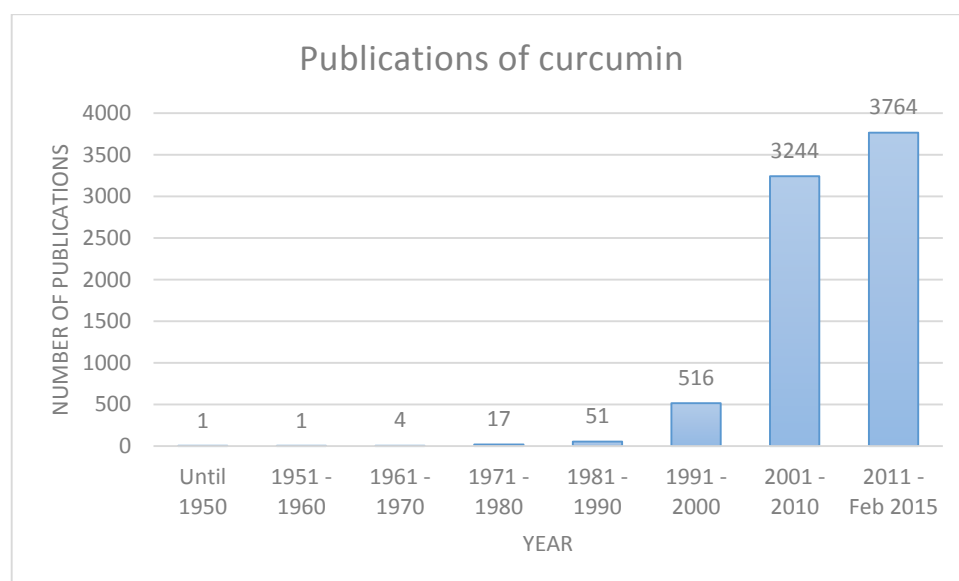
### 1.4.2 Main components in turmeric

Curcuminoids such as Curcumin I, Curcumin II and Curcumin III are major components which are 3-4 % of turmeric and their structures are illustrated in *Figure 9* (Chattopadhyay et al., 2004, Basnet and Skalko-Basnet, 2011). Among the three curcuminoids, curcumin I or simply called as curcumin is not only highly abundant compound but also biologically active one. Curcumin product that is available in the market is not pure curcumin but rather a mixture of curcuminoids (Basnet and Skalko-Basnet, 2011).



**Figure 9:** The three main curcuminoids presented in turmeric (Basnet and Skalko-Basnet, 2011).

The structure of curcumin was first identified in 1910 by Milobedzka and synthesized in 1913 by Lampe (Gupta et al., 2012). One of the first scientific papers showing pharmacological activity of curcumin was published in 1949 according to PubMed database, National Institute of Health. The interest on curcumin have been expanded since then and gained most interest markedly the past two decades which can be illustrated by the number of publication as shown in *Figure 10*. This is because of the promising therapeutic potentials shown by curcumin (Gupta et al., 2012). The pharmacological effects of curcumin are studied in animals as well as humans. Although based on PubMed database, different routes of administration such as orally, topically, intravenously and intraperitoneally were used, but the optimal therapeutic effects remained challenging due to its low solubility and poor bioavailability.



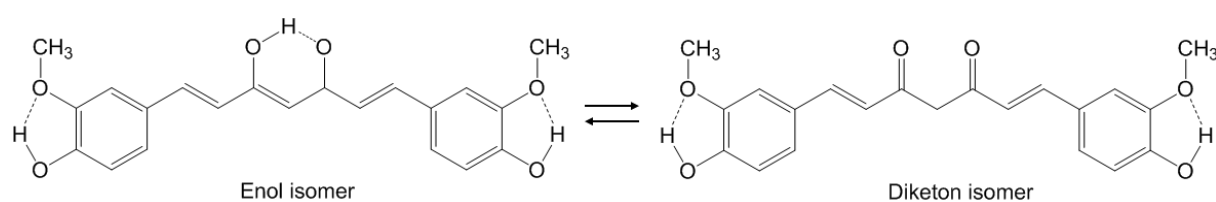
**Figure 10:** Number of publications on curcumin listed in PubMed database, National Institutes of Health (March 2015).

### 1.4.3 Chemical properties of curcumin

The general chemical characteristics of curcumin are mentioned in *Table 1*. Curcumin consists of two *para*- hydroxyl groups, two keto groups, two *ortho*- methoxy groups, an active methylene group and two double bonds (Pandey et al., 2011). Curcumin has two isomers, *enol*- and *keto*- form as shown in *Figure 11*.

**Table 1:** Chemical properties of curcumin

<b>Curcumin (curcumin I)</b>	
<b>IUPAC name:</b>	(1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (Shen and Ji, 2007)
<b>Molecular formula:</b>	C <sub>21</sub> H <sub>20</sub> O <sub>6</sub> (Padhye et al., 2010)
<b>Molecular weight:</b>	368,37 g/mol (Padhye et al., 2010)
<b>pKa:</b>	8,55 (Jovanovic et al., 1999)
<b>Maximum absorption peak (<math>\lambda_{\max}</math>):</b>	In methanol occurs at 420 – 430 nm (Sharma et al., 2005, Padhye et al., 2010)
<b>Bioavailability:</b>	Poor (Basnet and Skalko-Basnet, 2011)
<b>Solubility:</b>	Insoluble in water and ether. Soluble in acetone, dimethyl sulfoxide, ethanol, and methanol (Goel et al., 2008, Padhye et al., 2010, Gryniewicz and Ślifirski, 2012)
<b>Light sensitive:</b>	Yes (Sharma et al., 2005)



**Figure 11:** Molecular and optimized structure of curcumin in *enol*- and *keto*- form.

The structure of the *enol*- form is planar while the *keto*- form can be twisted in a *cis*- or *trans*-formation (Wright, 2002, Shen and Ji, 2007). *Enol*- form of curcumin is more stable than the *keto*- form, and the *cis*-diketo form is the most unstable of them all (Wright, 2002). It can be thought that because of the physiological pH, the *keto*- form will be dominating in cellular membranes (Jovanovic et al., 1999).

Curcumin has a strong visible yellow colour that comes from the two benzene-parts on each side of the carbon chain conjugation (Shen and Ji, 2007). The *enol*- form of curcumin dominates in basic pH and has a red colour, while the *keto*- form has a light yellow colour (Goel et al., 2008).

#### 1.4.4 Anti-oxidative activities of curcumin

Anti-oxidants in general are “compounds and reactions that dispose, scavenge and suppress formation of ROS or oppose their actions” (Bansal and Bilaspuri, 2010).

Anti-oxidants have three main functions (Agarwal et al., 2014):

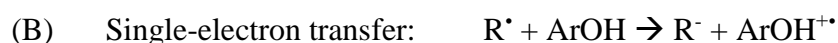
- 1) Works as chelators or binding proteins
- 2) Prevents formation of ROS
- 3) Remove ROS that are already present

Curcumin is polyphenolic phytochemical provided through diet. Most polyphenols have similar chemical nature showing anti-oxidative properties by the hydrogen atom transfer and single electron transfer mechanisms.

The hydrogen transfer mechanism is based on donating hydrogen atom, mostly from the phenol to free radicals as shown in equation A. This mechanism is dependent on bond dissociation energy (BDE) meaning that the lower BDE, the more efficient and rapid the donation will happen. The results of this mechanism are that the free radical are neutralized and phenoxy radical will be produced which is more stable and less reactive than the free radical (Quideau et al., 2011).



The second mechanism is the single-electron transfer where an electron from the phenol are transferred to a free radical as shown in equation B. This mechanism is dependent on ionization potential of the phenol.



Therefore, curcumin as a polyphenol compound is able to show anti-oxidant properties by both mechanisms as described above.

### **1.4.5 Safety and tolerability of curcumin**

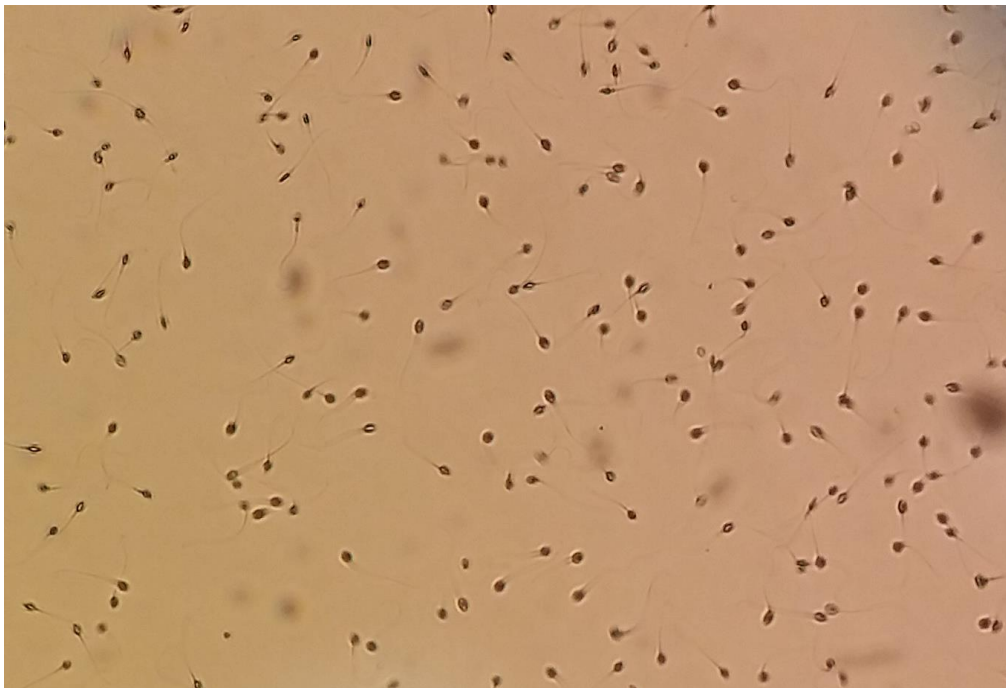
Curcumin has been taken daily in the form of curry spices (turmeric) up to 2 g per day daily in South Asian countries such as Nepal and India (Basnet and Skalko-Basnet, 2011). In the phase I clinical trial, up to 12 g per day orally administered curcumin was found non-toxic and tolerated (Lao et al., 2006, Shen and Ji, 2007). Goel et al. reported that toxic doses of curcumin is far above the therapeutic effective doses (Goel et al., 2008). Curcumin did not show teratogenic effects (World Health, 1974). Curcumin is considered safe and approved by FDA (Basnet and Skalko-Basnet, 2011).

Even though the studies showing high tolerability and safety of curcumin, it is important to keep in mind that none of the studies are performed directly so far in our knowledge on human sperm. The purpose of selecting curcumin for the current research aim was also due to its safety and tolerability profiles.

## 1.5 Semen

Half of the infertility is contributed by the male factor, mainly because of semen quality. The assisted reproductive technology (ART), mainly *in vitro* fertilization (IVF), has helped infertile/sub fertile couples to conceive baby. However there are further possibilities to develop ART by increasing semen quality.

During ART, germ cells are at risk for being exposed to high levels of ROS (Bansal and Bilaspuri, 2010). This can happen during microscopy because of exposure to light, different oxygen levels and temperature.

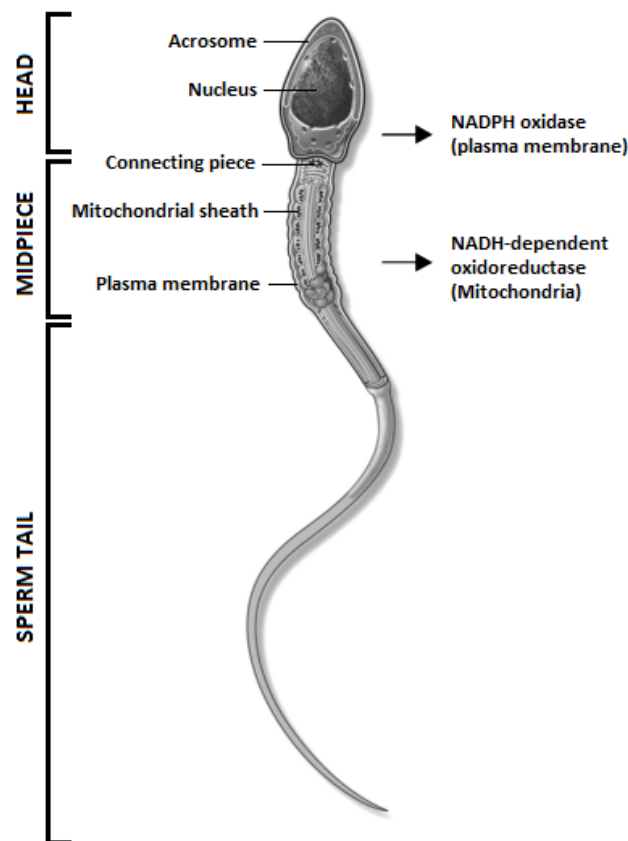


**Figure 12:** Picture of sperm cells observed under light microscope (x200).

Defective sperm functions are the most common cause of male infertility and difficult to treat. Among these, it is well known that oxidative stress contributes to the fertility status of sperm cells (Walczak–Jedrzejowska et al., 2013). Therefore by reducing the OS with supplement of anti-oxidants can improve the quality of the semen.

### 1.5.1 ROS and OS in human sperm cells

Spermatozoa also called sperm cells are male gametes. Sperm cell consists of head (5.1  $\mu\text{m}$  size), neck (7  $\mu\text{m}$  long) and tail (45  $\mu\text{m}$  long) (World Health, 2010) which are also shown in the light microscopic picture in *Figure 12*. A schematic diagram of a sperm cell is shown in *Figure 13*. Because of rapid movement of tail, it is a highly motile cell. Only motile sperm cells can bring fruitful fertilization, and therefore are the viability of the sperm cells expressed due to its motility. Sperm cells are rich in mitochondria because of its constant need for energy required for their rapid and constant movement. For electron transfer to happen in the electron transport chain, oxygen is required. Sperm cells have limited anti-oxidant mechanisms compared to other human cells. This is the reason to ROS accumulation in sperm cells leading to OS (Agarwal et al., 2003, Agarwal et al., 2014).



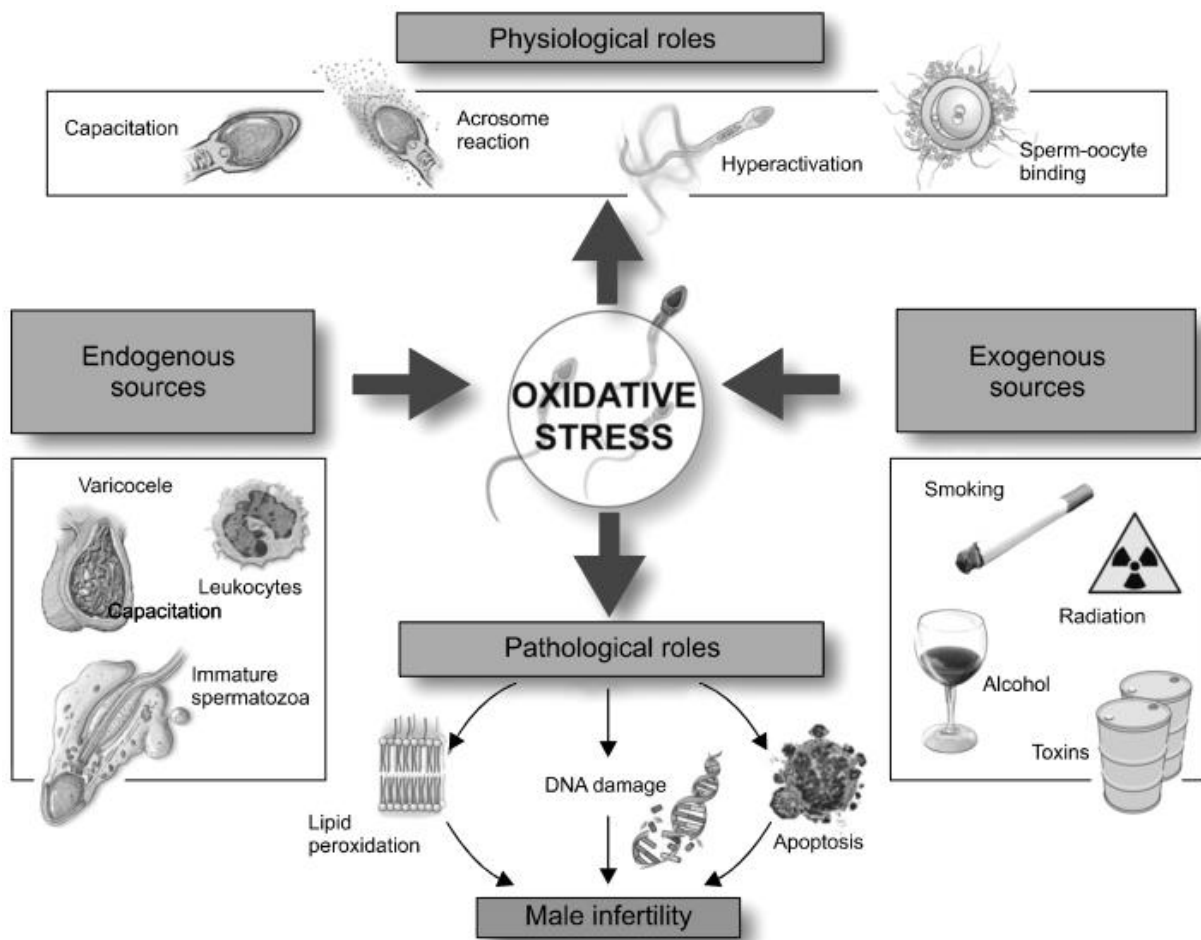
**Figure 13:** A modified figure of a sperm cell and two systems that generates reactive oxygen species (ROS) (Agarwal et al., 2014).

Sperm cells consist of intracellular lipid which contributes to energy production. By oxidizing these fatty acid chains, they have an important role as an energy source (Ahluwalia and Holman, 1969).

Two systems are involved in ROS generation in the human sperm cell as shown in *Figure 13* (Bansal and Bilaspuri, 2010, Agarwal et al., 2014);

- 1) The nicotinamide adenine dinucleotide phosphate oxidase system in sperm plasma membrane (NADPH oxidase)
- 2) The nicotinamide adenine dinucleotide-dependent oxidoreductase reaction in the mitochondria (NADPH-dependent oxidoreductase)

The anti-oxidant mechanism in sperm cells are limited. ROS have physiological functions and are necessary for normal process of cell proliferation, differentiation and migration. Despite the importance of ROS for the fertilization and other physiological functions, defective sperm functions are linked with OS produced by the excessive ROS production. And the defective sperm function induced by endogenously and exogenously produced ROS is the most common cause of male infertility as shown in *Figure 14* (Sharma and Agarwal, 1996, Gilbert and Colton, 2002, Bansal and Bilaspuri, 2010, Agarwal et al., 2014).



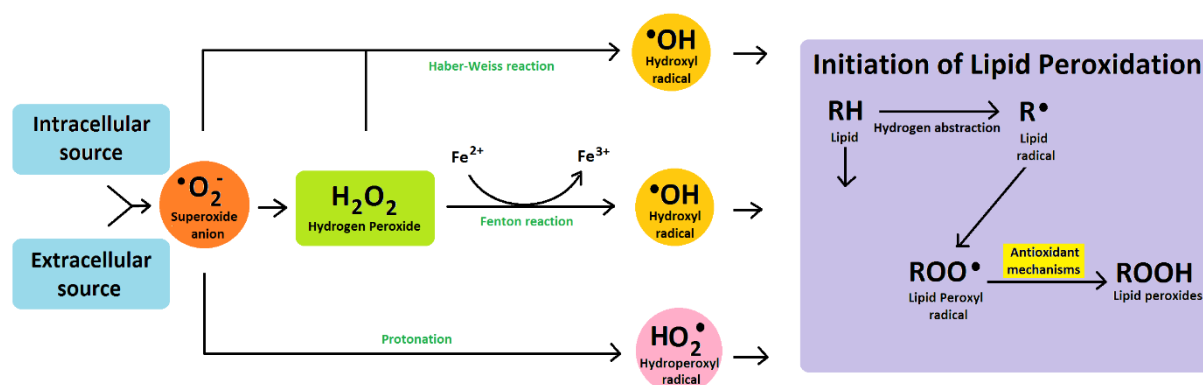
**Figure 14:** A schematic overview of oxidative stress produced in male reproductive system (Agarwal et al., 2014).



## 1.5.2 Extracellular anti-oxidant protection

Since the anti-oxidative mechanisms of sperm cells are less effective, can intake of some anti-oxidants like fruits, grains and vegetables have favourable effect (Halliwell, 1994, Gilbert and Colton, 2002, Bansal and Bilaspuri, 2010, Agarwal et al., 2014).

Literatures support that infertile men have higher levels of OS than fertile male, and infertile men have lower anti-oxidant levels in seminal plasma than fertile men. Supplement of anti-oxidants are therefore thought to have potentials in protecting against OS and can be used for the management of male infertility. Such anti-oxidant therapy usually involves oral anti-oxidant supplementation and *in vitro* supplementation in the culture medium in ART (Halliwell, 1994, Sharma and Agarwal, 1996, Gilbert and Colton, 2002, Bansal and Bilaspuri, 2010, Chen et al., 2013, Agarwal et al., 2014). As shown in *Figure 15*, free radicals or ROS are generated by inducing endogenous and exogenous sources. Hydroxyl lipid radicals can damage cellular function of the sperm cells. However, anti-oxidant can dilute the OS by strengthening cellular anti-oxidant systems and scavenging ROS or free radicals.



**Figure 15:** Summarized illustration of anti-oxidants scavenging ability on reactive oxygen species (ROS) and inhibition of lipid peroxidation.

Therefore, current research project is focused on a strong anti-oxidant, curcumin and to enhance its biological response, liposomal formulation were designed.



## 2 AIM OF THE STUDY

Application of nanotechnology in the field of medicine has been progressing rapidly. The therapeutic effects of the drugs associated with nanocarrier of the particle size (1-100 nm), differ comparing to conventional dosage forms. Recent advancement of nanotechnology based liposomal drug delivery are mainly improved: 1) the solubility of the drug, 2) the stability of the drugs, 3) the delivery to the targeted sites, and 4) the bioavailability and cellular uptake. The outcome was the optimal therapeutic effects with reduced side effects.

On the other hand, a part of infertility treatment and/or improvement of assisted reproductive technology (ART) can further be achieved by the improvement of semen quality. Due to the high metabolic rates and weak anti-oxidant systems, sperm cells are unable to defend themselves against the oxidative stress (OS) produced exogenously or endogenously. Vitamin C and/or vitamin E as anti-oxidants are supplemented in the culture media in the preparation of semen during IVF procedure. In spite of promising outcome, the clinical application has not been fully achieved. *It would be interesting to apply nanotechnology based liposomal delivery system for curcumin, a well-known anti-oxidant for the enhancement of motility and viability of human sperm cells in vitro.*

**Therefore, the main aim of the study was to develop curcumin incorporated in liposomal delivery system, and apply for the enhancement of motility and viability of human sperm cells *in vitro*.**

We hypothesize that liposomal formulation will improve curcumin solubility and cellular uptake by the sperm cells. Therefore, optimal and homogenous concentration of curcumin will be achieved inside and outside of the sperm cells. Like that, produced excessive ROS and OS can be controlled which may lead to the increased motility and viability.

Moreover, anti-oxidant capacity and effects of curcumin on the sperm motility and viability will be compared with that of vitamin C and vitamin E under the similar experimental conditions. This study will be a preliminary pilot study and carried out for the first time. Therefore, the main focus will be on the development of methods. The findings will serve as the base for further study improvement of semen quality by using nanotechnology based delivery systems.



## 3 MATERIAL AND INSTRUMENTS

### 3.1 Materials

ABTS, 2,2'-Azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Sigma Aldrich Co., St. Louis, USA

Alpha-tocopherol, Sigma-Aldrich, Steinheim, Germany

Ascorbic acid, Sigma-Aldrich Co., St. Louis, USA

Curcumin FLUKA Curcumin purum, Sigma-Aldrich, Steinheim, Germany

DMSO, Dimethyl sulfoxide, Sigma Aldrich chemie GmbH, Steinheim, Germany

DPPH, 2,2-Diphenyl-1-picrylhydrazyl free Radical (DPPH), Sigma-Aldrich®, St. Louis, USA

Ethanol, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Lipoid S100 (> 94 % phosphatidylcholine), Lipoid GMBH, Ludwigshafen, Germany

Methanol, Chromasolv®, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Methanol, E.Merck, Darmstadt, Germany

Methylene blue hydrate Ph.Eur., Sigma Aldrich, Steinheim, Germany

Modified HTF Medium, Irvine Scientific, Waalwijk, Netherlands

Potassium peroxodisulfate, Merck, Darmstadt, Germany

Quinn's Advantage® Protein Plus Fertilization (HTF), In-Vitro Fertilization Inc, Trumbull USA.

All other chemicals used were of analytical grade.

## 3.2 Equipment and instruments

Agilent 8453 UV-Visible Spectrophotometer, Agilent Technologies, Santa Clara, USA

Büchi Rotavapor R-124 with Büchi Vac V-500 Vacuum Pump System, Büchi Vacuum Controller B-721 and Büchi Water bath B-480, Büchi laborstechnik, Flawil, Switzerland.

Centrifuge Heraeus D-37520 Osterode Biofuge Pico, Heraeus Instruments, Germany  
Biofuge pico Heraeus Instruments, Germany.

Centrifuge Tubes with Screw Caps, VWR North America, USA

Conical Centrifuge Tubes, Thermo Fisher Scientific, New York, USA

Distillation unit Distinction D4000, Bibby Sterlin LDT, Staffordshire, UK

FALCON® 96-Well Cell Culture Plates, Corning Incorporated, New York, USA

Forma™ Series II 3110 Water-Jacked CO<sub>2</sub> Incubator, Thermo Scientific, USA

Improved Neubauer 0.100 mm depth, 0.0025 mm<sup>2</sup> counting chamber, Glaswarenfabrik Karl Hecht, Rhön, Germany

Leitz Fluovert FU inverted microscope, Wild Leitz GmbH, Germany

Menzel-Gläser British Standard Microscope slides, Thermo Fisher Scientific Inc., Waltham, USA

Menzel-Gläser Circle Cover Slips, Thickness 1.5, Thermo Fisher Scientific Inc., Waltham, USA

NICOMP Submicron particle sizer, model 370, Nicomp Particle sizing systems, Santa Barbara, California, USA.

Nucleopore® Track-Etched Membranes (PC) Polycarbonate, size 0.8 µm, 0.4 µm, 0.2 µm, 0.1 µm, Whatman International Ltd., Maidstone, UK.

Open-top UV quartz Cell cuvette, 10 mm, 3.0 ml volume, Agilent Technologies, Germany.

SONICS High Intensity Ultrasonic Processor, 500 Watt Model with Temperature Controller, probe diameter 13 mm, Sonics & Materials Inc., Newtown, USA.

SuperClear® Centrifuge Tubes with Plug Style Caps, Labcon North America, USA

VITREX micro-haematocrit tubes, VITREX Medical A/S, Herlev, Denmark

Vortex Genie2™, Bender & Hobeinag, Zürich, Switzerland.

Zeiss 10x and 40 x objective lenses, Carl Zeiss, West Germany

Zeiss Standard Binocular Microscope, Carl Zeiss, West Germany

Zetasizer Nano Zen 2600, Malvern instruments Limited, Engima Business Park, Grovewood Road, Malvern, Worcestershire, UK.

### **3.3 Computer programs**

Büchi Rotavapor R-124 with Büchi Vac V-500 Vacuum Pump System with Büchi Vacuum Controller B-721 and Büchi Water bath B-480, Büchi, Switzerland

NICOMP Particle Sizing Systems, CW388 Application Version 1.68, Santa Barbara, California, USA.

NICOMP Submicron particle sizer, model 370, Nicomp Particle sizing systems, Santa Babara, California, USA

SONICS High Intensity Ultrasonic Processor, 500 Watt Model with Temperature Controller, probe diameter 13 mm, Sonics, Newtown, USA

UV-Visible ChemStation Software B.04.02, Agilent Technologies 2001-2011, Germany.

Vortex Genie2™, Bender & Hobeinag, Zürich, Switzerland

Zetasizer Nano Zen 2600, Malvern instruments Limited, Engima Business Park, Grovewood Road, Malvern, Worcestershire, UK

Zetasizer software version 7.03, Malvern Instrument Limited, Malvern, UK.



## 4 EXPERIMENTAL SECTION

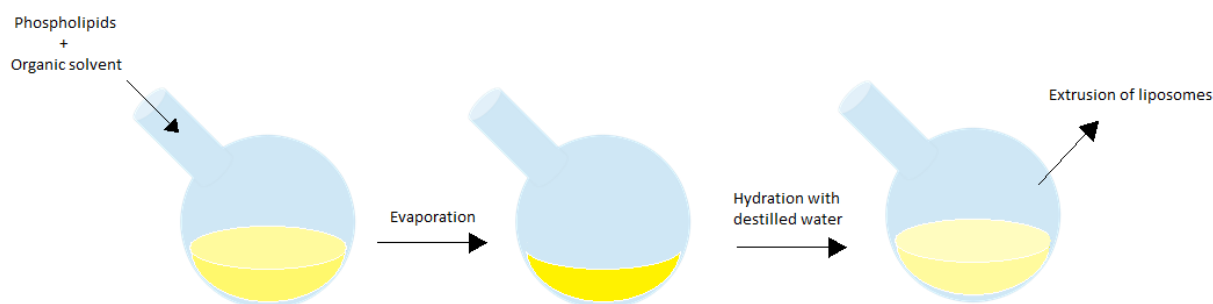
### 4.1 Preparation of liposomes

#### 4.1.1 Preparation of empty liposomes

Lipoid S100 (200 mg) was weighted in a 100 ml round bottom flask and dissolved in sufficient methanol. A Büchi Rotavapor R-124 with Büchi Vac V-500 Vacuum Pump System, Büchi Vacuum Controller B-721 and Büchi Water bath B-480 (Büchi laborotechnik, Switzerland) were used to hydrate the lipid. The set up was adjusted to 50 mmHg, 45 °C and 70 rpm. The rotation was adjusted to 94 rpm when all the organic solvent evaporated, and when the pressure was around 60 mmHg, the flask was immersed deeper into the water bath. By optimal pressure, the lipid film evaporated in one hour and the potential traces of solvent were removed by flushing with nitrogen gas for one minute. Lipid film was resuspended quickly in 10 ml distilled water, and a vortex was used to mix properly and dislodge all film from the flask bottom and walls. The liposomes were stored in the refrigerator for 12 hours before further experiments.

#### 4.1.2 Preparation of liposomes with curcumin

Curcumin (20 mg) and Lipoid S100 (200 mg) were weighted, dissolved in methanol, evaporated and liposomes resuspended in distilled water as shown in *Section 4.1.1*. Most of the lipids were removed from the flask wall by using a vortex and ultrasonic bath for one minute to remove lipid from the flask bottom. Curcumin loaded liposomes were stored in the refrigerator for 12 hours before further experiments.



**Figure 16:** Preparation of liposomes

## **4.2 Liposomal characterization**

### **4.2.1 Size reduction of liposomes**

#### *4.2.1.1 Sonication*

SONICS High Intensity Ultrasonic Processor 500 Watt Model with Temperature Controller (Sonics & Materials Inc., USA) was used to reduce the size of liposomes. Liposomal suspension was transferred to a 10 or 20 ml beaker and put into an ice bath. The probe was positioned in the centre of the suspension, without touching the bottom or the glass walls. The suspension was exposed to ultrasound with an output of 50 Watt for continuous 2 minute cycles to achieve the desired particle size of 100 nm. The liposomes were stored in the refrigerator at 4 °C for 24 hours before further analysis.

#### *4.2.1.2 Extrusion*

Nuclepore® polycarbonate membranes (Whatman International Ltd., UK) at sizes 800, 400, 200 and 100 nm were used as filter for extrusion of liposomes. Liposomes (10 ml) were first vortex for 2 minute before being transferred to a syringe (10 ml). The liposomes were extruded through the filter five times for each respective pore size. After extrusion, the liposomes were placed in the fridge for minimum 2 hours before further experiments.

### **4.2.2 Vesicle size determination**

A NICOMP Submicron particle sizer model 370 with Helium-Neon diode laser operating at 632.8 nm (NICOMP, USA) was used to determine the liposome sizes and distribution. Test tubes were rinsed with distilled water and afterward put in an ultrasonic bath for 10-15 minute. The sample preparation was made in laminar airflow bench to avoid possible contamination with dust particles. Test tubes were cleansed with fresh filtered distilled water (0.22 µm pore size syringe filter) before the suspension was diluted to an intensity of 250-350 kHz (Ingebrigtsen and Brandl, 2002), before the measurement. Each suspension was measured for 3 times 10 minute cycles at a fixed angle of 90° to the incident light. The results were presented as intensity weight.

### **4.2.3 Zeta potential determination**

A Zetasizer Nano Zen 2600 (Malvern, UK) was used to measure zeta potential. The cell was cleansed with 96 % ethanol and rinsed with filtrated tapped water. One ml syringes were used to clean and fill the cell with suspension. The suspension was prepared by diluting liposomes 1:20 (v/v) with filtrated tapped water. The measurement was set to 3 cycles and 100 runs.

### **4.2.4 Spectral analysis**

A stock solution of curcumin and 96 % ethanol with concentration 100 000 ng/ml was prepared. The stock solution was gradually diluted until a spectrum with maximum absorbance peak ( $\lambda_{\text{max}}$ ) around 2 was measured in Agilent 8453 UV-vis spectrophotometer with deuterium and tungsten lamp using an open-top UV quartz Cell cuvette (10 mm, 3,0 ml volume) (Agilent Technologies, USA). The limit of spectral analysis was at 12 500 ng/ml concentration.

### **4.2.5 Standard curve of curcumin**

Absorbance was measured on a spectrophotometer as described in *Section 4.2.4*. A standard curve was made from a stock solution at 100 000 ng/ml diluted to the concentrations 1000, 800, 600, 400, 200 and 100 ng/ml with 96 % ethanol. The measuring wavelength was set to 425 nm. A standard curve was made by plotting absorbance against the concentration of standard solutions.

### **4.2.6 Separation of un-incorporated curcumin**

Extruded liposomal suspensions were centrifuged on Heraeus Biofuge Pico centrifuge (Heraeus Instruments, Germany) to separate liposomes from un-incorporated curcumin. The centrifugation had a speed at 1000 g for 10 minute. The pellet with un-incorporated curcumin and supernatant with liposomal curcumin were separated, weighted and volume was determined by pipetting.

#### **4.2.7 Entrapment efficiency**

Entrapment efficiency was measured with a spectrophotometer as in *Section 4.2.4*. Each liposomal suspension was first disintegrated with 96 % ethanol before diluting to a measurable absorbance. The entrapment efficiency was measured by the following equation:

$$\text{Entrapment efficiency (\%)} = \frac{A}{(A + B)} * 100$$

A: amount of curcumin in supernatant, B: amount of curcumin in pellet

#### **4.2.8 Stability testing**

The stability of empty liposomes and liposomes incorporated with curcumin were determined by measuring the vesicle size after 5 months of storage in 4 °C. The measurements were performed as described in *Section 4.2.2*.

### **4.3 Anti-oxidative activities**

Anti-oxidative activities is expressed as the capacity of curcumin, vitamin C and vitamin E to scavenge chemically generated free radicals such as 2,2'-Azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) diammonium free radical (ABTS<sup>++</sup>), and 2,2-Diphenyl-1-picrylhydrazyl free radical (DPPH<sup>•</sup>) by the standard methods (Basnet et al., 1997, Re et al., 1999).

#### **4.3.1 Preparation of ABTS<sup>++</sup> and DPPH<sup>•</sup> solution**

The ABTS<sup>++</sup> solution was prepared by mixing 1:1 (v/v) ABTS solution (7.4 mM) with potassium peroxydisulfate (PPOS) solution (2.6 mM) and diluted four times with 96 % ethanol.

DPPH<sup>•</sup> solution (120 μM) was prepared by solubilizing DPPH with 96 % ethanol.

#### **4.3.2 Spectral analysis of ABTS<sup>++</sup> and DPPH<sup>•</sup>**

A spectral analysis were carried out by mixing 300 μl of free radical solution (ABTS<sup>++</sup> or DPPH<sup>•</sup>) with 300 μl 96 % ethanol. The absorbance was measured in a Agilent 8453 UV-vis spectrophotometer with deuterium and tungsten lamp using an open-top UV quartz Cell cuvette (10 mm, 750 μl volume) (Agilent Technologies, USA).

#### **4.3.3 Determination of anti-oxidant activities of vitamin C, vitamin E and curcumin by ABTS or DPPH radical scavenging assay**

ABTS<sup>++</sup> and DPPH<sup>•</sup> solution were made as described in *Section 4.3.1*. Vitamin C (10 mM) was prepared by solubilizing ascorbic acid with distilled water. One mM solution was prepared mixing 10 mM vitamin C solution 1:10 (v/v) with 96 % ethanol. Vitamin E (1mM) and curcumin (1 mM) solution were prepared by solubilizing with ethanol.

Each anti-oxidant solution were diluted to the concentrations 1, 5, 10 and 20 μM. ABTS<sup>++</sup> or DPPH<sup>•</sup> solution were mixed 1:1 (v/v) with each anti-oxidant and left in dark at room temperature (24 °C) for 30 minute. The anti-oxidative activities of curcumin, vitamin C and vitamin E were carried out at absorbance 750 nm for ABTS and 519 nm for DPPH assays, respectively. The measurements were carried out in an UV-Vis spectrophotometer. All experiments were performed with three separate measurement.

## 4.4 Semen preparation

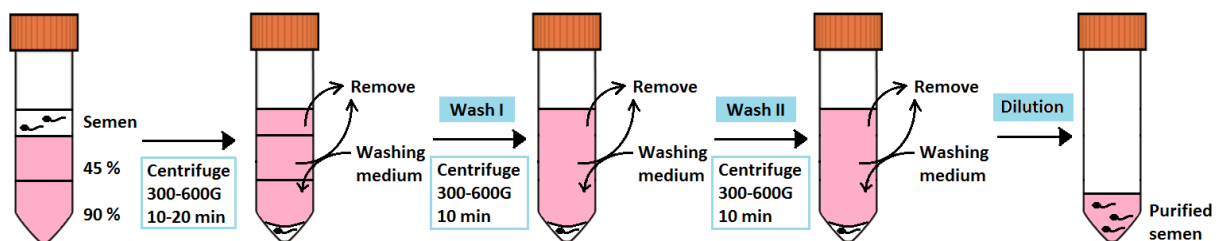
The project protocol was approved by the Norwegian Regional Committee for Medical Research Ethics North Norway (REK nord: 2014/932). Written informed consent was obtained from all patients included in the study.

### 4.4.1 Fresh semen

Semen samples were collected from the IVF patient at the IVF clinic at the University Hospital of North Norway (UNN) or from healthy men of age 20 to 40. Only semen samples having good motility and sperm concentration were used for the motility and viability enhancement experiments. The semen samples were collected from men who abstained ejaculation for a minimum of 3 to 5 days. The person carried experiments were blinded to the patient history. The detection of sperm quality were based on aggregation, concentration, morphology, motility, motility grade, pH, viscosity and volume after the criteria according to WHO guidelines (World Health, 2010).

### 4.4.2 Purified semen – The density gradient centrifugation method

Semen sample was purified by the density gradient centrifugation method according to the guidelines at IVF clinic at UNN. Briefly, semen samples were freshly collected in the hospital. It was liquefied for 30 minute at room temperature with slow shaking. Semen sample (1 to 1.5 ml) was applied in the upper layer (45 %) and lower layer (90 %) gradient solution each 1.5 ml in a 12 ml test tube. The samples were centrifuged 300-600 g in 10-20 minute. Two top layers or supernatant were discarded. Only purified semen with good quality from the bottom layer were collected and washed with sperm wash medium twice.



**Figure 17:** A schematic drawing of density gradient centrifugation method.

The residue of the sperm cells was homogenized in a swim up medium containing human tubular fluid (HTF). After insemination at 13:00 at the IVF-clinic in UNN, the remaining samples were collected and used for the motility and viability enhancement experiments.

## **4.5 Characterization of sperm cells**

### **4.5.1 Total count concentration of sperm cells**

Semen samples (fresh or purified) were diluted 10 or 20 times (v/v) with methylene blue in a test tube and left for 10 minute. A capillary tube (VITREX Medical A/S, Denmark) was used to transfer some of the homogenized and diluted semen suspension over to an improved 0.1 mm, 0.0025 mm<sup>2</sup> depth counting chamber (Glaswarenfabrik Karl Hecht, Germany). The counting chamber was placed into the moist environment in a petri dish for 10 minute before total count was determined with the help of a Zeiss Standard Binocular microscope (Carl Zeiss, West Germany). Sperm cells were diluted to  $1 \times 10^5$  cells/ml for the experiment later on.

### **4.5.2 Sperm motility and motility grade**

Motility and motility grade were determined after observing around 100 sperm cells in the inverted microscope (Wild Leitz, Germany). The categories of sperm movement were graded:

0: no movement

1: movement in a located area with no forward movement

2: movement in small circles

3: good movement either linearly forward or in large circle

4: quick movement either linearly forward or in large circle

## 4.6 Preparation of anti-oxidant solutions and liposomal suspensions

### 4.6.1 The effect of saline and swim up HTF medium on human sperm cells

Saline (0.9 % NaCl) were diluted to the final concentrations; 0, 8, 113, 135, 169, 203, 225, 338, 405, 450, 675 and 810  $\mu\text{g/ml}$  with HTF medium.

### 4.6.2 Dilution of anti-oxidant solutions and liposomal suspensions

Two methods of dilutions were used to find the effect of anti-oxidants, empty liposomes and liposomal curcumin on human sperm cells.

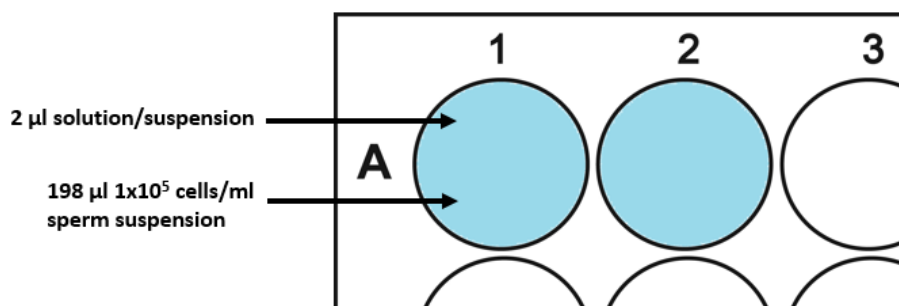
#### 4.6.2.1 Method 1: Sperm viability

Liposomes were prepared as previously described in *Section 4.1*. Stock solutions of 2, 2, 10 20  $\text{mg/ml}$  of lipid in empty liposomes and liposomal curcumin were prepared. Each of the stock solutions were diluted 10, 100, 200, 400 and 600 times with swim up medium containing HTF.

Each diluted liposomal suspension (2  $\mu\text{l}$ ) were added into 198  $\mu\text{l}$  of  $1 \times 10^5$  sperm cells/ml in a FALCON® 96-Well cell culture plates (Corning Incorporated, USA) (*Figure 18*) with two replicates.

The final concentrations were of lipid were: 0.03, 0.05, 0.08, 0.10, 0.13, 0.17, 0.20, 0.25, 0.50, 1, 2, 5, 10, 20  $\mu\text{g/ml}$ . The curcumin-lipid ratio was 1:10 (w/w).

The plates with sperm and liposomal suspension were incubated in a Forma™ Series II 3110 Water-Jacked CO<sub>2</sub> incubator (Thermo Scientific, USA) for 24 hours in 37 °C and 5 % CO<sub>2</sub> atmosphere before observation in inverted microscope as described in *Section 4.5.2*.



**Figure 18:** Method 1: Preparation of semen suspension for tolerance and toxicity determination.

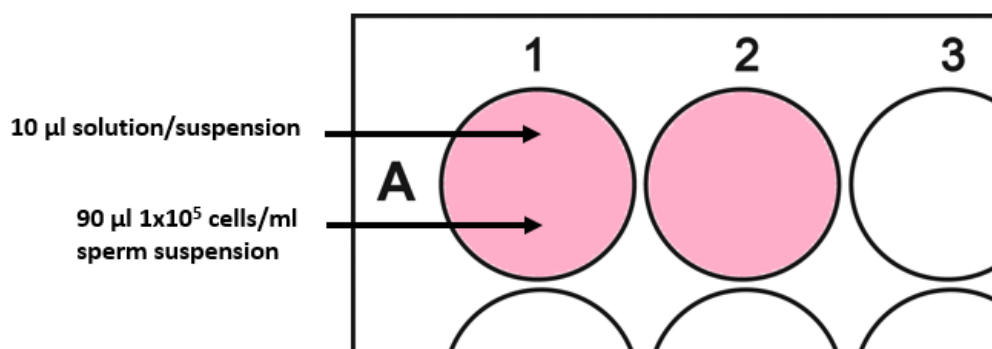


#### 4.6.2.2 Method 2: Sperm viability

Stock solution of empty liposomes and liposomal curcumin were prepared as previously described in *Section 4.1*. Each lipid suspension (10  $\mu$ l) were diluted to 1 ml in fertilization medium to prepare 200 ng/ $\mu$ l. Each of the suspensions were diluted to 20, 40, 200 and 400 times with fertilization HTF medium to obtain final concentration as 10, 5, 1 and 0.5  $\mu$ g/ml. Each suspension (10  $\mu$ l) was added into 90  $\mu$ l sperm cell suspension with  $1 \times 10^5$  sperm cells/ml concentration.

Stock solution of curcumin, ascorbic acid and  $\alpha$ -tocopherol each with 10 mg/ml, were made in DMSO (curcumin and vitamin E) or HTF medium (vitamin C). Each stock solution of the anti-oxidant was diluted 1, 2, 10 and 20 times with fertilization medium containing HTF to obtain final concentration as 10, 5, 1 and 0.5  $\mu$ g/ml.

Anti-oxidant solution (10  $\mu$ l) was added to 90  $\mu$ l of  $1 \times 10^5$  cells/ml sperm cell suspension (*Figure 19*) and incubated at same condition as previously described (4.6.2.1).



**Figure 19:** Method 2: Preparation of semen cell suspension for tolerance and toxicity determination.

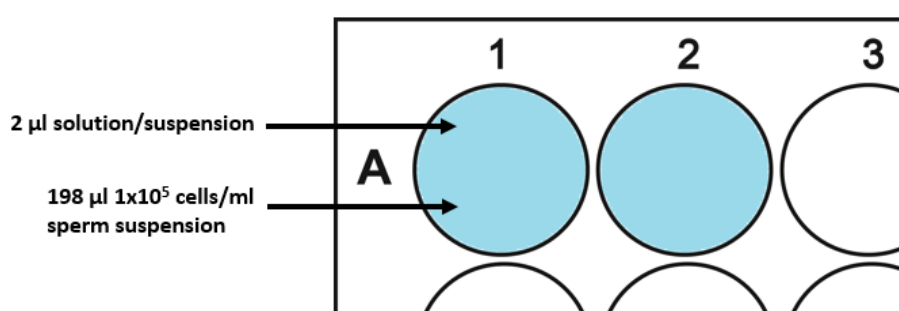
#### 4.6.3 Sperm motility and viability with anti-oxidants and liposomes

Stock solutions of empty liposomes and liposomal curcumin were prepared as previously described in *Section 4.1*. Stock solutions of curcumin and  $\alpha$ -tocopherol were prepared by solubilizing in DMSO, and ascorbic acid with water.

**Table 2:** A presentation of stock and final anti-oxidant solutions and liposomal suspensions

	<b>Empty liposomes</b>	<b>Liposomal curcumin</b>	<b>Free curcumin</b>	<b>Ascorbic acid</b>	<b>A-tocopherol</b>
<b>Stock concentration</b>	20 mg/ml	2 mg/ml (5.4 $\mu$ M)	2 mg/ml (5.4 $\mu$ M)	2.30 mg/ml (5.4 $\mu$ M)	0.95 mg/ml (5.4 $\mu$ M)
<b>Final concentration</b>	0.40 $\mu$ g/ml	0.04 $\mu$ g/ml (0.1 nM)	0.04 $\mu$ g/ml (0.1 nM)	0.05 $\mu$ g/ml (0.1 nM)	0.02 $\mu$ g/ml (0.1 nM)

Every stock solution were diluted 500 times with HTF. Anti-oxidant solution or (2  $\mu$ l) was added 198  $\mu$ l of  $1 \times 10^5$  cells/ml sperm suspension in 96-Well cell culture plate (*Figure 20*). Fresh semen as described in *Section 4.4.1* was used in this experiment. It was carried out two replicates.



**Figure 20:** Preparation of semen suspension for determination of sperm motility and viability.

The suspension was prepared 2-4 hours after hand in from donor. The plates with suspension were incubated in a Forma<sup>TM</sup> Series II 3110 Water-Jacked CO<sub>2</sub> incubator (Thermo Scientific, USA) for around 24 hours in 37 °C and 5 % CO<sub>2</sub> atmosphere before observation in the inverted microscope as described in *Section 4.5.2*.

## 4.7 Statistical methods

All the results are expressed as mean  $\pm$  SD from at least two or three independent experiments. The significance of the variability was determined with student t-test for the comparison of two or three means if possible. A p value  $\leq 0.05$  were considered statistically significant.

## 5 RESULTS AND DISCUSSION

Current project was focused on the development of liposomal formulation as carrier system for anti-oxidant destined for enhancing sperm cells motility and viability. Therefore, a simple but important anti-oxidant, curcumin, was selected for optimize its liposomal formulation. It is a well-known fact that oxidative stress in the sperm cells is induced by the free radicals produced endogenously or exogenously (*Figure 14*). In this respect, curcumin is one of the widely discussed and clinically relevant substance due to its free radical scavenging properties. Therefore, it was also necessary to examine the anti-oxidative activity of curcumin compared to vitamin C and vitamin E and their effect on the sperm cells motility and viability before optimization of liposomal formulations with curcumin.

### 5.1 Liposomal formulation

For the preparation of empty liposomes, approximately 200 mg lipids were used. In case of liposomal curcumin, approximately 200 mg of lipid and 20 mg of curcumin were used. Each formulation was prepared twice separately and summarized in *Table 3*. In all preparation curcumin-lipid ratio was 1:10 (w/w).

**Table 3:** The contents of lipid and curcumin in different liposomal suspensions

	<b>Lipoid S100 (mg)</b>	<b>Curcumin (mg)</b>
<b>Empty liposomes</b>	204 ± 1.4	-
<b>Liposomes with curcumin</b>	201 ± 1.4	20.15 ± 0.1

The values are presented as mean ± SD (n = 2).

The size distributions can be presented by Gaussian distribution which measures a monomodal distribution, while NICOMP distribution measures a multimodal distribution of particle sizes. Sometimes, the vesicle sizes have multimodal distribution. In this case, calculating the mean size of the whole distribution gives a very inaccurate size and the NICOMP function should be used instead. Chi square ( $\chi^2$ ) is used to describe if the observed size distribution are fit with the expected distribution. If the  $\chi^2$  is between 0-2, the Gaussian analysis should be used. A value over 3 should use the NICOMP analysis (Ingebrigtsen and Brandl, 2002).

Sonication reduces MLVs to oligolamellar liposomes and SUVs through pulsed input of high energy (New, 1990). Extrusion is an easy, effective and gentle method to reduce liposomal size (Spuch and Navarro, 2011). Extrusion through polycarbonate membranes in general results in vesicles with a mean size smaller than the pore size in the membrane filter (New, 1990).

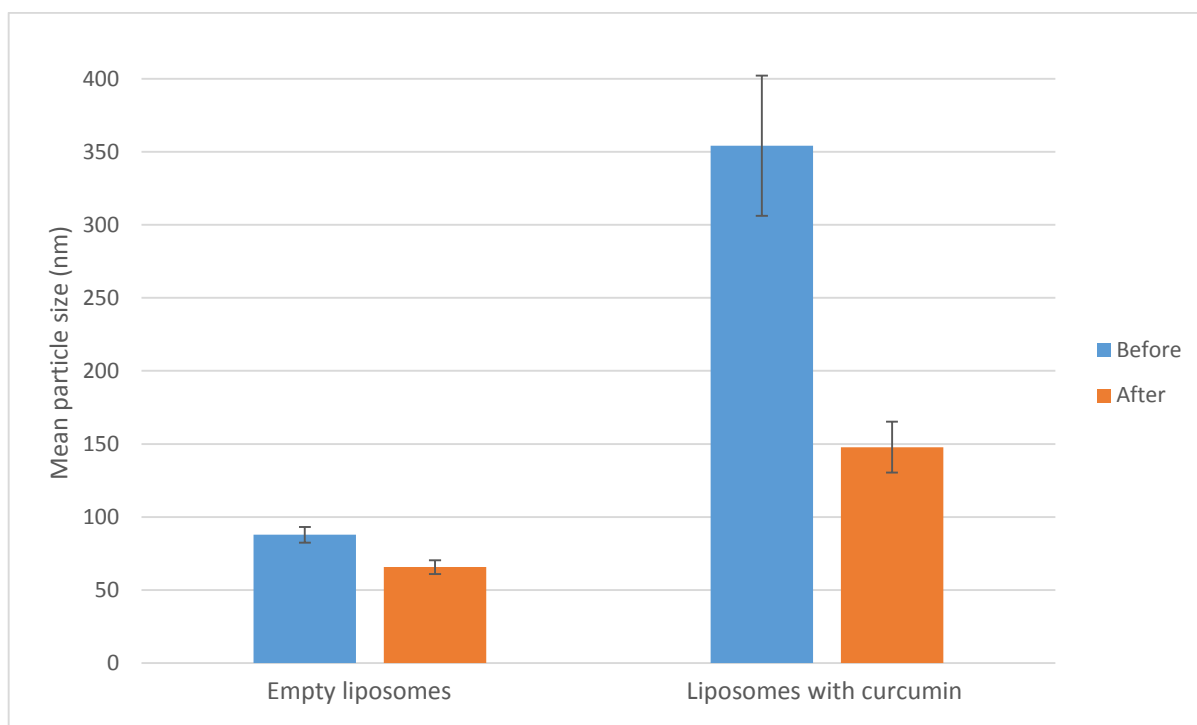
In our case, the original liposomes prepared by the film method before sonication or extrusion had vesicle size around 1  $\mu\text{m}$ . The sonication reduced the vesicle sizes of empty liposomes and liposomal curcumin significantly, however the polydispersity values were rather high. It was therefore decided to use extrusion method instead and results are presented in *Table 4* are for the extruded liposomes.

**Table 4:** Characterization of liposomes

	<b>Particle size (nm)</b>	<b>Polydispersity index (PI)</b>	<b>Entrapment efficiency</b>	<b>Zeta potential (mV)</b>
<b>Empty liposomes</b>	65.68 $\pm$ 4.7	0.3 $\pm$ 0.0	-	-1.78 $\pm$ 0.1
<b>Liposomes with curcumin</b>	147.82 $\pm$ 17.3	0.2 $\pm$ 0.1	88.8 $\pm$ 2.3 %	-3.83 $\pm$ 0.8

The values are presented as the mean  $\pm$  SD (n = 2).

Extrusion reduced the vesicle sizes to 65.68  $\pm$  4.7 nm for empty liposomes and 147.82  $\pm$  17.3 nm for liposomes with curcumin (*Table 4*). The vesicle size of the extruded empty liposomes was smaller than the membrane pore size of 100 nm used in the extrusion which is in agreement with literature (New, 1990). However, for liposomes with curcumin, the vesicles were not smaller than 100 nm (*Figure 21*). This was somehow expected since we experienced the difficulties reducing these liposomes by sonication as well. It is evident that incorporation of curcumin in liposomal bilayers makes the bilayers more rigid resisting the size reduction. Curcumin is a relatively big molecule (MW 368) (*Table 1*) (Padhye et al., 2010). It consists of two benzene rings and has a conjugated structure which gives a planar structure. The conjugates make the molecule rigid forming more rigid bilayer membrane compared to a pure phospholipid bilayer membrane. The branch-structure of curcumin in its planar or bend shape is thought to be space consuming (Barry et al., 2009), explaining why liposomes with curcumin were larger than empty liposomes.



**Figure 21:** Mean vesicle size of liposomes before and after extrusion.

The values are presented as the mean  $\pm$  SD (n = 2).

In this case, the  $\chi^2$  of empty liposomes ranged from 31.5 to 96.7 which indicates multimodal distribution of particle sizes. The NICOMP analysis should have been used instead of the Gaussian analysis. Taking in consideration that around 77 % of the vesicles in the empty liposomes had sizes around 96 nm as shown in *Table 5*, the Gaussian analysis were decided to be used so it could be compared to liposomal curcumin. It was considered that the amount of vesicles around 26 nm could not have big influence on the results, even though the mean size of empty liposomes in *Table 4* in theoretical should be bigger. However, the size of the empty liposomes are still under 100 nm which were the preferred size for this experiment.

**Table 5:** The vesicle sizes of empty liposomes based on the NICOMP analysis.

<b>Peak 1: Mean vesicle size (nm)</b>	<b>Peak 1: Percentage (%) of total distribution</b>	<b>Peak 2: Mean vesicle size (nm)</b>	<b>Peak 2: Percentage (%) of total distribution</b>
26.18 $\pm$ 3.8	22.65 $\pm$ 4.3	96.25 $\pm$ 20.3	77.36 $\pm$ 4.2

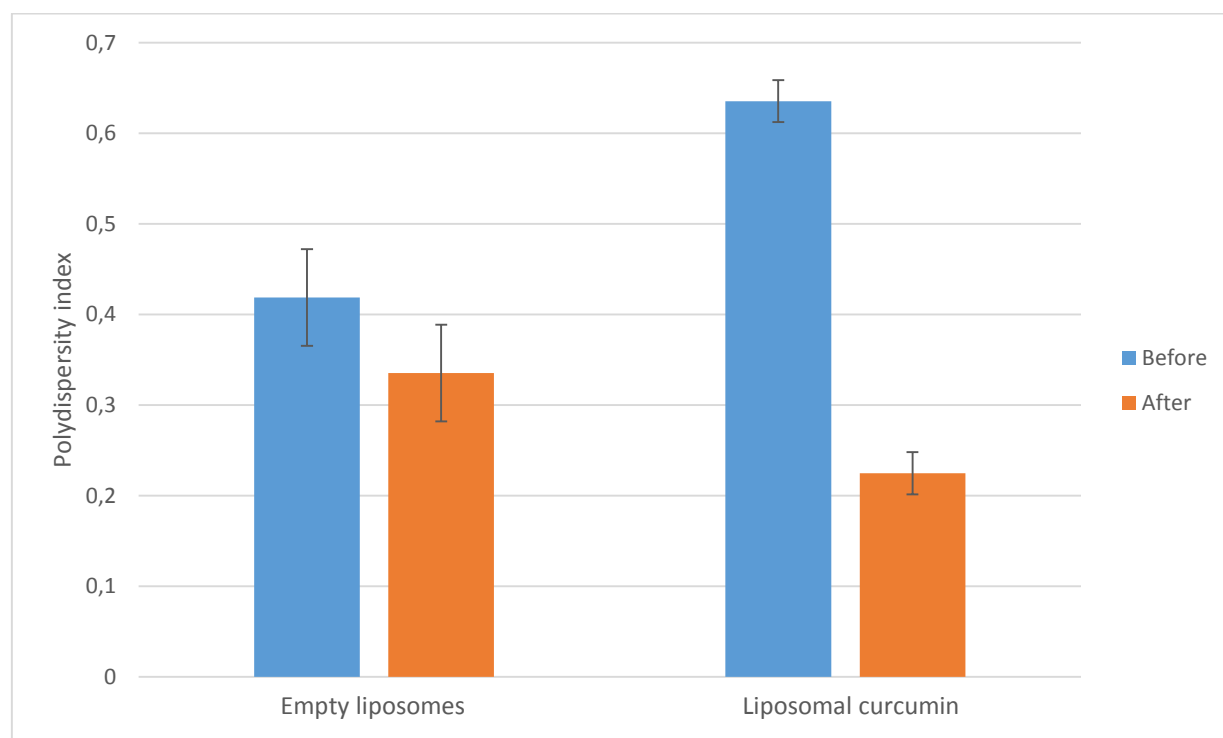
The values are presented as mean  $\pm$  SD (n=2).

The high energy transferred to the liposomal curcumin during sonication lead to over 50 % volume loss of suspension (data is not shown). This was another reason why the extrusion method was preferred in the current project. Extrusion gave around 20 % suspension loss, which was considerable a lot less.

**Table 6:** Volume loss of liposomal curcumin during extrusion.

<b>Volume of liposomal curcumin before extrusion</b>	20 ml
<b>Volume of liposomal curcumin after extrusion</b>	15.5 ml
<b>Volume lost during extrusion</b>	4.5 ml
<b>Percentage lost volume during extrusion</b>	22.5 %
<b>Theoretical amount of lost curcumin during extrusion</b>	4.5 mg

The polydispersity index (PI) is used to describe the distribution of vesicle sizes, where PI over 0.3 in this case was considered as a broad distribution, and PI under 0.3 is considered a homogenous size distribution. The empty liposomes had a PI around 0.3 and 0.2 for liposomes with curcumin as shown in *Figure 22* and *Table 4*.



**Figure 22:** The effect of extrusion on the polydispersity of liposomes.

The values are presented as the mean  $\pm$  SD (n = 2)

Sonicated empty liposomes were compared to extruded empty liposomes to see if the size reduction methods had an influence on the polydispersity of the vesicles. The vesicles were significantly ( $p = 0.01$ ) more uniform for the extruded liposomes as shown in *Table 7*.

**Table 7:** The effect sonication and extrusion have on the polydispersity of empty liposomes

	<b>Probe sonicated liposomes</b>	<b>Extruded liposomes</b>
<b>Polydispersity index</b>	$0.4 \pm 0.1$	$0.3 \pm 0.0$

The values are presented as mean  $\pm$  SD ( $n = 2$ ).

Curcumin is suggested to be inside the phospholipid layer of liposomes and contributes to ordering the membrane. The interaction happens through hydrogen bonding with the choline group in the head of phospholipid and van der Waals bindings between the aromatic rings with the phospholipid acyl chains (Barry et al., 2009).

The entrapment efficiency of curcumin in liposomes were found to be 89 % as shown in *Table 4*. This was slightly higher, but similar to the findings as previously reported (Hussain, 2010). In his work he applied sonication as the intention to reduce vesicle size, whereas we used the extrusion. The ratio between the lipid and curcumin also differed between two projects. In another study by Basnet et al., it was found EE to be  $82.1 \pm 3.2$  % and  $67 \pm 5.9$  % for liposomes with starting amount of curcumin at 5 and 10 mg (Basnet et al., 2012). The equation used for calculating EE in the study was different from the one used in this experiment. When the EE was recalculated to the same equation as Basnet et al., EE was found to be  $59.2 \pm 7.8$  %. This finding supports that the EE of curcumin decreased when higher amount of curcumin are added during the preparation and that actual values are best presented as microgram of curcumin per mg of lipid.

Hussain also described that recovery of the liposomal curcumin was between 80 and 90 % (Hussain, 2010). In our case, the recovery was found to be  $66.5 \pm 7.1$  % which is much lower, but can be explained. After the extrusion, the total amount of curcumin in the liposomal suspension was no longer 20 mg because some curcumin was lost during the extrusion.

Since the remaining volume after extrusion was about 22.5 % less than the original (*Table 6*), recalculated recovery was  $81.3 \pm 16.1$  %. This supports the findings of Hussain even though we applied different methods to reduce the vesicle sizes. However, in this experiment it was

assumed that the volume loss only lead to curcumin loss. It was not taken considerations to lipid loss which can have happened.

The expected zeta potential for the liposomes was zero because the compounds (phosphatidylcholine and curcumin) used in the liposomal preparation were not charged or zwitterionic (Pasenkiewicz-Gierula et al., 1999). The slightly negative zeta potential at  $-1.78 \pm 0.1$  for empty liposomes and  $-3.83 \pm 0.8$  for liposomal curcumin (*Table 4*) were supported by Andersen et al. (Andersen et al., 2015). The small difference in zeta potential are due to experimental set up or maybe lipid oxidation to air (Fiume, 2001). The slightly negative zeta potential was not taken in account during further testing of the SUVs because slightly negative surface of liposomes tend to interact better with cells and increases endocytosis by target cell (Allen and Cullis, 2013). Since the purpose of this thesis is to see if the uptake of curcumin improves, the slightly negative charge was considered favourable.

Studies show that curcumin has poor bioavailability because of limited absorption, rapid metabolism and rapid clearance and elimination from the body (Sharma et al., 2005, Anand et al., 2007, Wang et al., 2014). Poor bioavailability is one of the reasons why the potential of curcumin could not be fully utilized (Gupta et al., 2012). Nanotechnology was used for the purpose of changing the poor solubility and homogenous distribution of curcumin molecules as can be observed by naked eye (*Figure 23*).



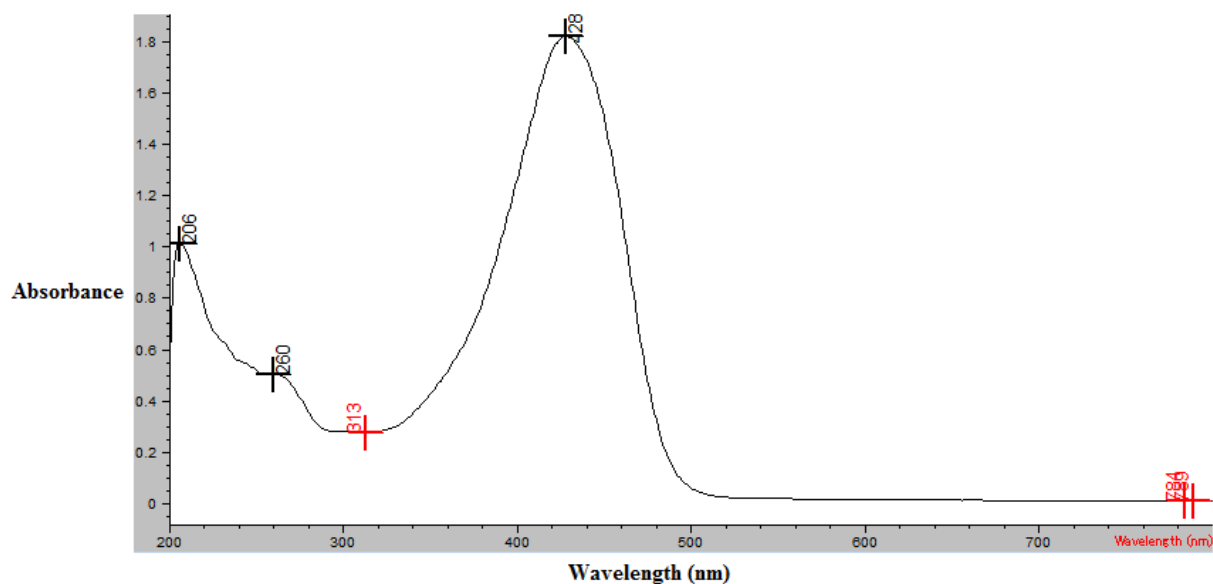
**Figure 23:** Bottle to the left contains curcumin in water. Bottle to the right contains curcumin entrapped in liposomal suspension in water.



Higher cellular uptake is observed when the solubility increase. The increased solubility of curcumin was observed after incorporation in liposomes as shown in *Figure 23*. The bottle on the left contains a particular amount of curcumin mixed with water which could not be solubilized even after constant shaking. After using nanotechnology by incorporating the curcumin in liposomes, the curcumin was distributed homogenously, giving a homogenous suspension.

## 5.2 Spectral analysis of curcumin

The quantitative estimation of curcumin was carried out by UV-Vis methods taking curcumin as standard. To understand the maximal absorbance peak of curcumin ( $\lambda_{\max}$ ), a spectral analysis of curcumin was performed (*Figure 24*).



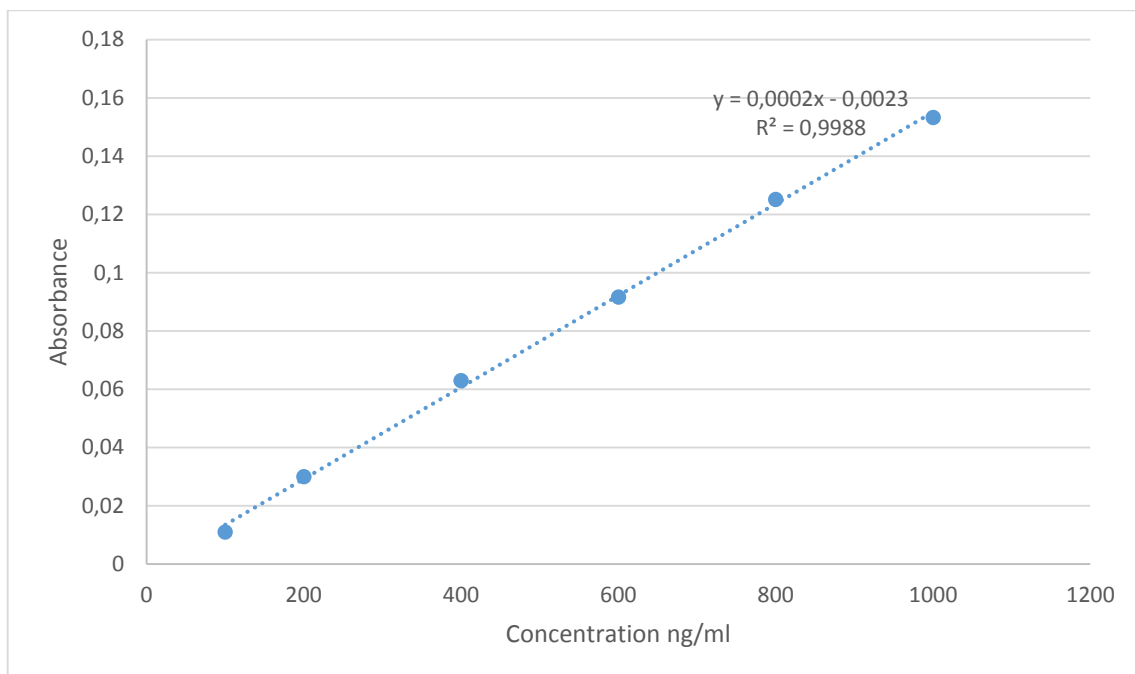
**Figure 24:** UV-Vis spectrum of curcumin dissolved in 96 % ethanol.

The spectral analysis gave a maximum absorption peak ( $\lambda_{\max}$ ) around 428 nm as shown in *Figure 24*, indicating that absorbance of curcumin in between 420-430 nm can be used for the quantitative analysis (Shen and Ji, 2007, Goel et al., 2008, Padhye et al., 2010).

### 5.3 Standard curve of curcumin

Based on Beer-Lamberts law, the absorbance is proportional to a substance concentration which can absorb electromagnetic radiation. In order to quantify entrapment efficiency of curcumin, the amount of entrapped curcumin in the liposomal vesicles had to be quantified. For this purpose, a standard curve of curcumin was needed. By measuring the absorbance of a test sample knowing the absorbance in different concentrations, it is possible to use determine the concentration of the sample by using Beer-Lamberts law (Grøn, 2005).

The standard curve for curcumin was prepared by measuring  $\lambda_{\text{max}}$  at the wavelength of 425 nm as shown in *Figure 25*. A standard curve with good correlation ( $R^2 = 1.00$ ) was obtained. The equation ( $y = 0.0002x - 0.0023$ ) was used to calculate the curcumin amount in the liposome.



**Figure 25:** Standard curve of curcumin in 96 % ethanol. The small standard deviations are not shown in the graph.

## 5.4 Stability testing of liposomes with curcumin

To determine if vesicle size and polydispersity of liposomes with curcumin changed during the storage, two liposomal suspensions were measured after 5 months at 4 °C (*Table 8*). The mean diameter of the vesicles and polydispersity seemed smaller after the storage than before. However, student t-test was performed and showed no significant differences ( $p = 0.32$ ). This indicated that the liposomes with curcumin were stable during storage, which is important parameter in the optimization of their characteristics.

**Table 8:** Comparison of vesicle sizes of liposomes with curcumin after 5 months of storage in refrigerator at 4°C.

Mean diameter of fresh sample	Polydispersity index (P.I)	Mean diameter after 5 months	Polydispersity index (P.I)
161.17 ± 17.46	0.10 ± 0.01	154.77 ± 16.71	0.09 ± 0.01

The values are presented as mean ± SD (n = 2) with three replicates.

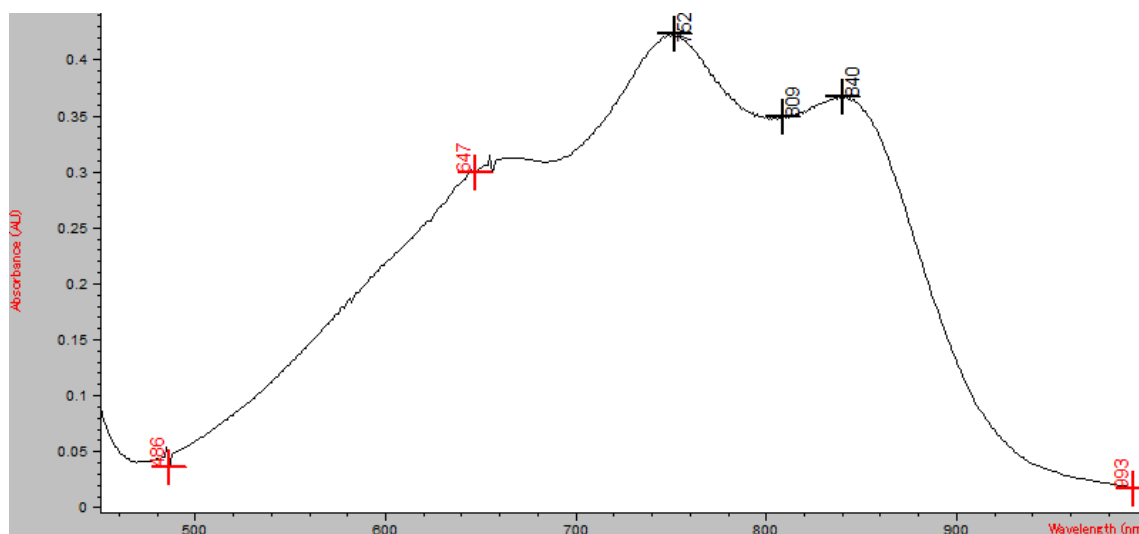
## 5.5 Anti-oxidative activities of curcumin, vitamin C, vitamin E

Anti-oxidative activities of curcumin was expressed as the capacity to scavenge the stable free radicals generated in the laboratory chemically. For this purpose cationic free radical ABTS (ABTS<sup>•+</sup>) and neutral free radical DPPH (DPPH<sup>•</sup>) were used. The concentration of ABTS<sup>•+</sup> and DPPH<sup>•</sup> is directly correlated to deep green and deep violet colour, respectively. Therefore the concentration can be measured by UV-Vis spectrophotometer. The chemical reactions of these radicals towards anti-oxidants are almost equivalent to those of the biologically generated free radicals such as super oxide, nitric oxide, peroxy nitrite and hydroxyl radicals (Kedare and Singh, 2011).

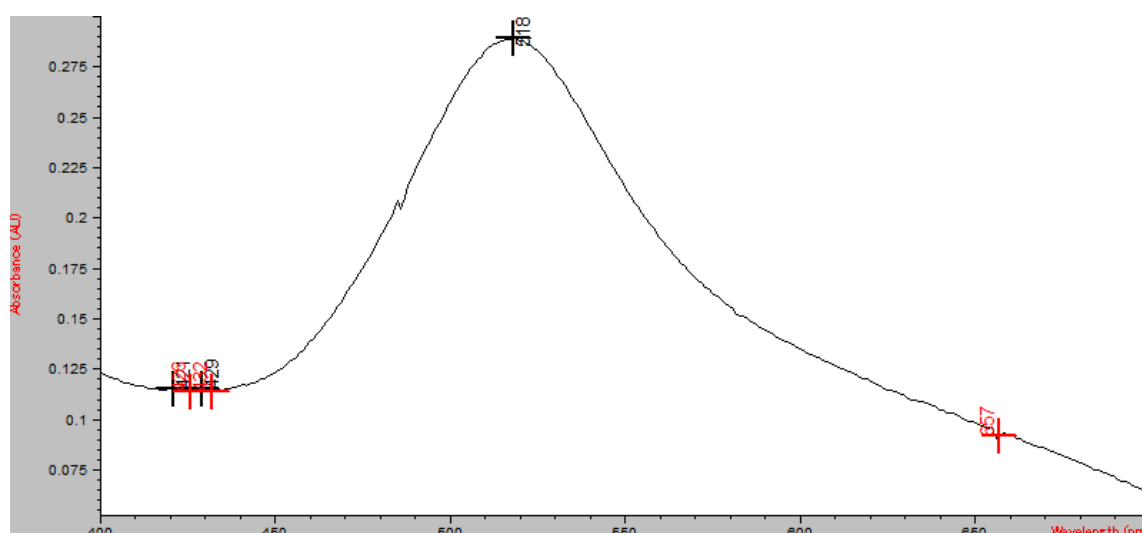
When ABTS<sup>•+</sup> or DPPH<sup>•</sup> are mixed with anti-oxidants, these radicals are easily reduced to ABTS or DPPH which are colourless. Based on this principle, ABTS<sup>•+</sup> and DPPH<sup>•</sup> solutions are widely used methods to determine the anti-oxidative activities of different substances because of its accuracy, easiness and inexpensiveness (Re et al., 1999, Erel, 2004, Kedare and Singh, 2011).

### 5.5.1 Spectral analysis of ABTS<sup>•+</sup> and DPPH<sup>•</sup>

A complete spectra of ABTS<sup>•+</sup> or DPPH<sup>•</sup> radical solutions were measured (*Figure 26* and *Figure 27*). Both spectra were measured taking 96 % ethanol as blank and all sample solution were diluted in the ethanol. Complete UV-Vis spectra of ABTS<sup>•+</sup> or DPPH<sup>•</sup> radical solutions revealed the ideal concentration of the free radicals and  $\lambda_{\max}$  position which were important to know before anti-oxidant reaction. Spectral analysis, can help to decide and select absorbance for the quantitative measurements without UV-Vis crossover interferences.



**Figure 26:** UV-Vis spectrum analysis of ABTS<sup>•+</sup> in 96 % ethanol.



**Figure 27:** UV-Vis spectrum analysis of DPPH<sup>•</sup> in 96 % ethanol.

The spectral analysis of the ABTS<sup>•+</sup> showed the maximal absorbencies ( $\lambda_{\max}$ ) at 647, 752 and 840 nm as shown in *Figure 26*. For DPPH<sup>•</sup>,  $\lambda_{\max}$  was found at 518 nm as shown in *Figure 27*. The maximum absorbance measured for curcumin is at 428 nm (*Section 5.2*), for  $\alpha$ -tocopherol at 295 nm and for ascorbic acid at 260 nm (Karpińska et al., 2005, National Center for Biotechnology Information, 2015a). Therefore, anti-oxidative activity were measured for ABTS<sup>•+</sup> and DPPH<sup>•</sup> solutions at 750 nm and 519 nm, respectively.

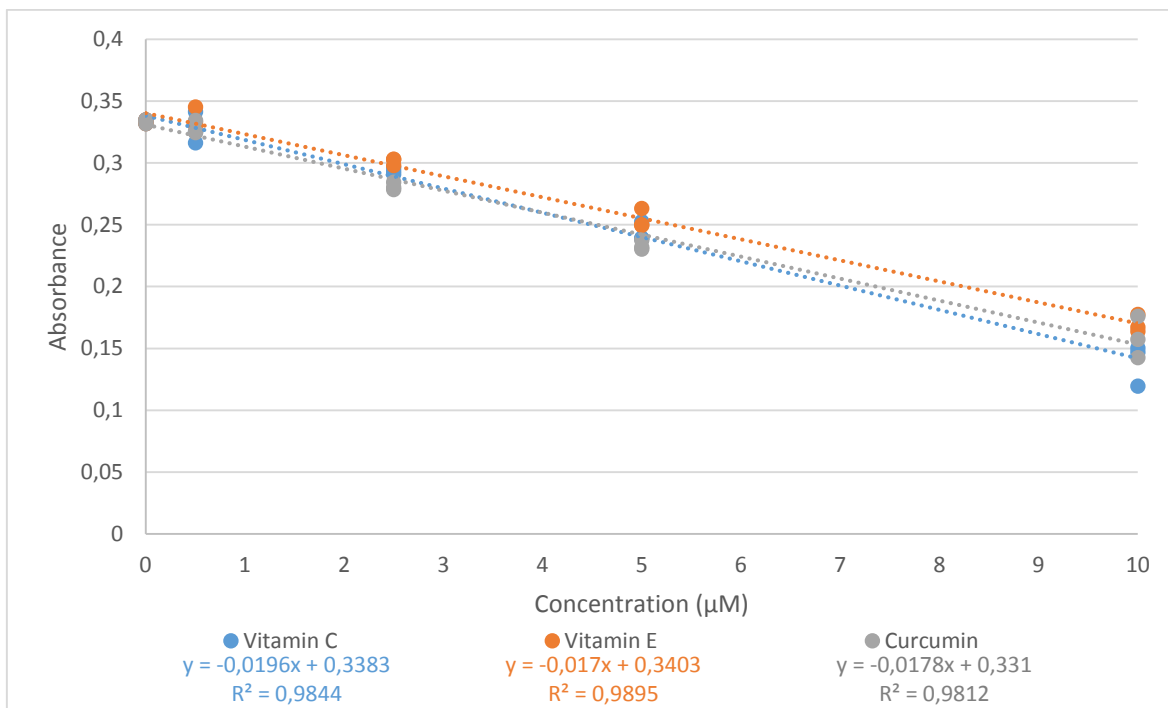
### **5.5.2 Free radical scavenging capacity of vitamin C, vitamin E and curcumin**

Curcumin was tested for its anti-oxidative activities comparing to ascorbic acid and  $\alpha$ -tocopherol because of their known anti-oxidative activities. The chemical structure of curcumin also resemble ascorbic acid and  $\alpha$ -tocopherol (Buettner, 1993).

Curcumin is similar to  $\alpha$ -tocopherol structurally because of the phenolic ring with a long carbon-chain like curcumin. Instead of two phenolic rings on each end as curcumin,  $\alpha$ -tocopherol has only one. The molecule weight of  $\alpha$ -tocopherol (MW: 430) is closer to curcumin (MW 368) (National Center for Biotechnology Information, 2015a)

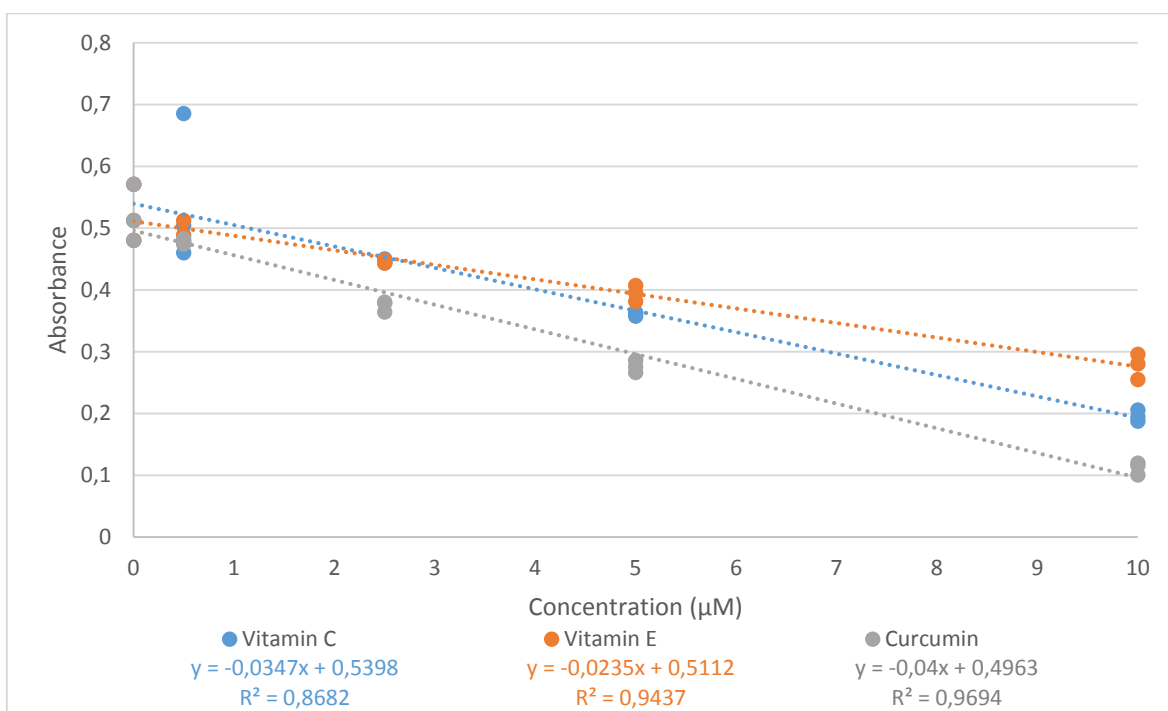
Ascorbic acid consists of a furan ring. The molecular size of ascorbic acid (MW: 176) is much smaller than curcumin (National Center for Biotechnology Information, 2015b). However, both vitamin C and curcumin can scavenge free radicals with almost similar potential (*Figure 28* and *Figure 29*).

Free radical scavenging capacity of vitamin C, vitamin E and curcumin were measured at 0.5, 2.5, 5 and 10  $\mu$ M concentration under similar conditions. All three anti-oxidants showed the concentration dependent radical scavenging activities. The results are shown in *Figure 28* and *Figure 29*. The higher the concentrations were of the substances, the more free radicals were scavenged as can be seen with the decreased free radicals of ABTS and DPPH.



**Figure 28:** DPPH radical scavenging activities of ascorbic acid,  $\alpha$ -tocopherol and curcumin in 96 % ethanol.

The regression equations for DPPH radicals scavenging activity for vitamin C and vitamin E and curcumin are shown in *Figure 28*. In this experiment, vitamin C showed slightly steeper slope indicating that vitamin C had the strongest anti-oxidative activity among the three.



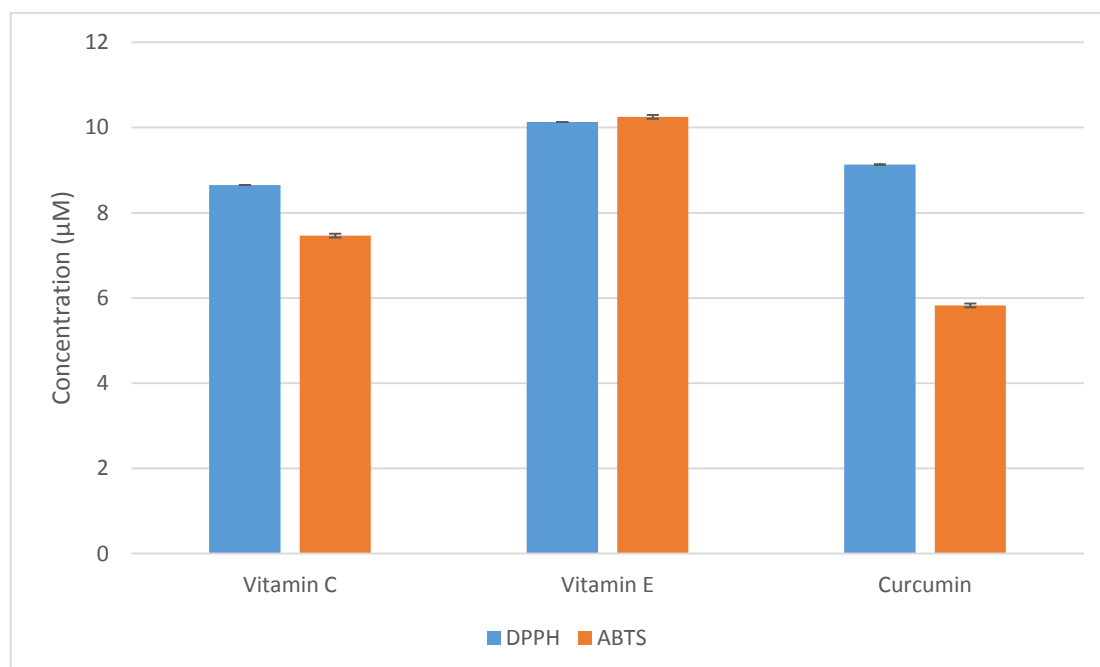
**Figure 29:** ABTS radical scavenging activities of ascorbic acid,  $\alpha$ -tocopherol and curcumin in 96 % ethanol.

The regression equations for ABTS radicals scavenging activity for curcumin, vitamin C and vitamin E are shown in *Figure 29*. It was found that the slope for curcumin was the steepest of all the slopes in the ABTS solution, indicating curcumin is strongest ABTS radical scavenger. Ak and Culcin reported that  $\alpha$ -tocopherol had a steeper slope than curcumin in both ABTS<sup>+</sup> and DPPH<sup>•</sup> solution (Ak and Gulcin, 2008). It should be noted that the anti-oxidant activity are comparatively similar for all three anti-oxidants.

A student t-test were performed in this experiment to see if there was a significant difference among curcumin, vitamin C and vitamin E for their anti-oxidative activity. The results showed no significant difference between the anti-oxidants ( $p \geq 0.12$ ) giving no grounds to say if one anti-oxidant is better than another. This experiment confirmed that all the three anti-oxidants were very potent radical scavenger.

### 5.5.3 Half maximal effective concentration of vitamin C, vitamin E and curcumin

The half maximal effective concentration ( $EC_{50}$ ) describes the concentration of substrate that expresses 50 % reduction of free radical as measured by decreased colour intensity. The lower  $EC_{50}$  mean higher radical scavenger activity.

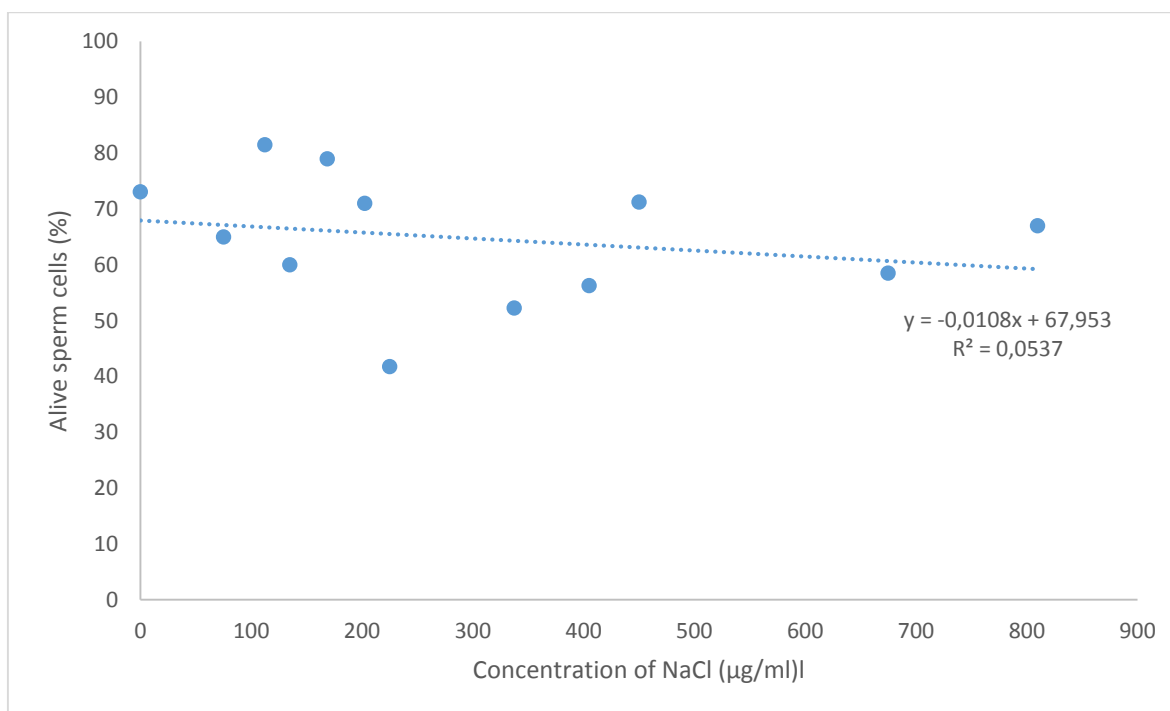


**Figure 30:** The half maximal effective concentration ( $EC_{50}$ ) in DPPH<sup>•</sup> and ABTS<sup>+</sup> solution.

The EC<sub>50</sub> for curcumin were 5.82 μM for ABTS<sup>++</sup> solution and 9.13 μM for DPPH<sup>•</sup> solution as shown in *Figure 30*. The results are very similar to the previously published results (Basnet et al., 2012). Ak and Gulcin reported that the EC<sub>50</sub> for curcumin to be 34.86 μg/mL (94.63 μM) in DPPH and 18.07 μg/mL (49.05 μM) in ABTS for curcumin. The EC<sub>50</sub> in this study are roughly 10 times higher than what was found in our experiment (Ak and Gulcin, 2008). It might be due to the purity of samples or dilution errors but our results are reproducible and similar to several other published results.

## 5.6 The effect of saline and HTF on human sperm cells

The effect of various dilutions of saline and swim up medium containing HTF was studied to find the optimal condition for controls. Motility and viability is highly reduced if only saline is used (data is not shown). As saline concentration was reduced and swim up medium-HTF alone was used, a constant viability was observed. The results in *Figure 31* showed that swim up medium-HTF did not affect the sperm viability within 24 hours after various sperm cell concentrations.



**Figure 31:** The effect of saline and swim up medium on sperm viability *in vitro*.



It was seen that the trend was slightly decreased when proportion of saline increased (*Figure 31*). A-t-test were used to determine if there was a significant difference in longevity for high and low concentrations of saline.

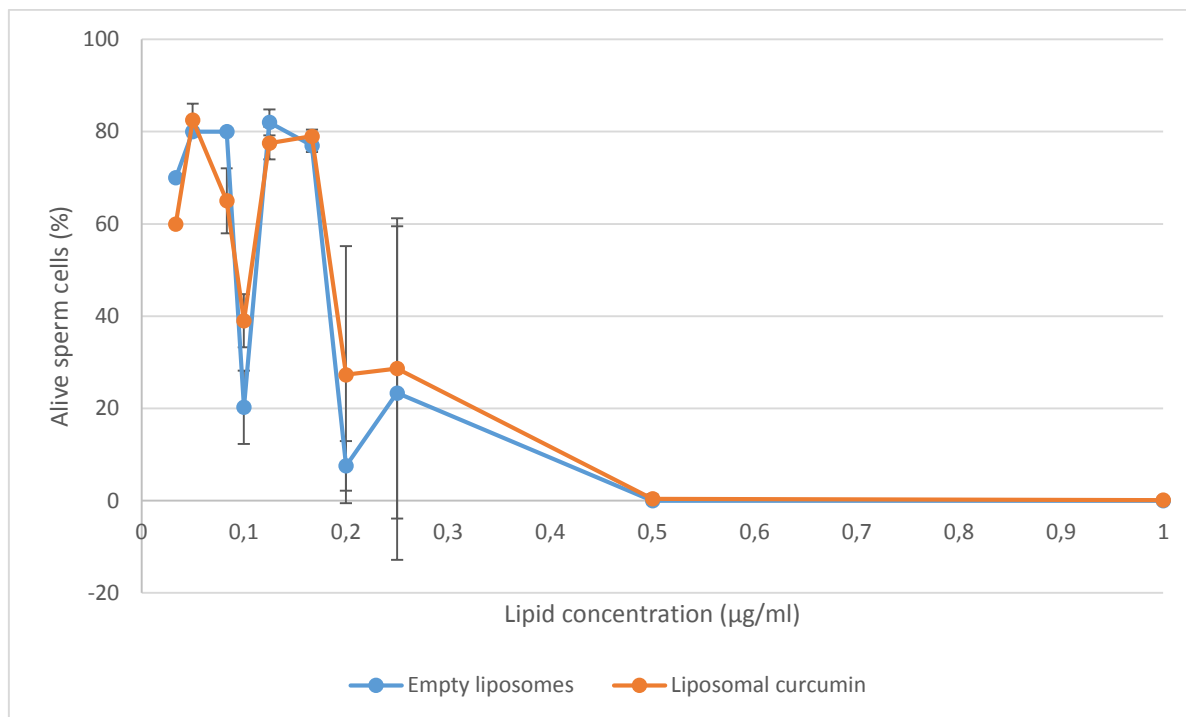
The concentrations 0 to 405 µg/ml were compared to the concentrations 405 to 810 µg/ml, and no significant difference were found ( $p = 0.85$ ). Since the proportion of HTF medium are proportional to concentration of NaCl, this indicated that lower saline did not affect the viability of sperm cells. However, only swim up medium-HTF were chosen to be used for further experiments.

## **5.7 Effects of anti-oxidants and liposomal curcumin on sperm cells**

One of the main purposes of the current research work was to establish the methodology. As stated previously, the objective of the study was to observe whether liposomal formulations show the *in vitro* enhanced effects on the motility and viability to the sperm cells. Three well known anti-oxidants, curcumin, vitamin C and vitamin E were selected for the study. These anti-oxidants were directly applied to the sperm cells taking dissolving solvents as the controls. In addition, curcumin was incorporated into liposomal vesicles to prepare liposomal curcumin. To observe the effects of liposomal curcumin, it was compared with the effect of empty liposomes taking as the controls. More over the effects of the liposomal curcumin were also compared with that of the directly applied anti-oxidants. Therefore, several independent methods were carried out based on hit-and-trial. At present, two separate methods are performed.

### **5.7.1 Method 1: Sperm viability**

Without knowing the therapeutically effective concentration of curcumin on sperm cells, it was carried out different dilutions of empty liposomes and liposomal curcumin to find the optimal concentrations. Details of the dilutions were carried out as shown in *Section 4.6.2.1*. The results are expressed after 24 hours of exposure of liposomal curcumin comparing to control with only swim up medium-HTF (*Figure 32*).



**Figure 32:** The effect of liposomal curcumin on sperm viability *in vitro*.

When empty liposomes were diluted with HTF medium, the viability of sperm cells raised drastically as shown in *Figure 32*. Low concentrations of lipid seemed to have highest viable sperm cells. When the concentration increased, the viability was observed decreased to concentrations 0.5 µg/ml. In concentrations higher than 0.5 µg/ml showed no viability at all.

For empty liposomes, around 0.4 µg/ml reduced less than 90 % viability as shown in *Figure 32*. Moreover as the concentrations were at 0.2 µg/ml or lower, more than 30 % sperm cells were alive.

Similar results were found for the liposomal curcumin (*Figure 32*). In this case of liposomal curcumin, the amount of lipids were the same as the empty liposome but in addition to lipid, curcumin was added. A t-test showed no significant difference ( $p = 0.069$ ) for empty liposome and liposomal curcumin.

It is unlikely that soy lecithin, which consists of phosphatidylcholine (PC) was found toxic at 0.5 µg/ml concentration to sperm cells. Soy lecithin is confirmed safe by the World Health Organization (WHO) and have been awarded Generally Recognized as Safe (GRAS) status by the US Food and Drug Administration (FDA) (World Health, 1974, Fiume, 2001). Therefore a detailed study to explain the teratogenic effects is needed.

Few safety studies have been carried out on sperm cells, but PC concentrations up to the 100 µg/ml have found no impact on sperm motility. PC have instead been found to have important functions for the acrosomal reaction (Cross, 1994). Lecithin which consists of PC has also shown effective supplement to reduce risk of contamination during sperm freezing (Jeyendran et al., 2008).

In an article by Hong et al., it was found that PC concentration from 100 to 3000 µM showed no significant effect on human sperm motility (Hong et al., 1986). In this experiment, the toxic concentrations for empty liposomes were found at 0.5 µg/ml (approximately 0.66 µM) PC. An explanation of this can be the liposomal formulation. In Hong's experiment, the PC were dissolved in chloroform, evaporated under nitrogen to prepare film of lipid in the test tube. It might be due to the lipid concentration were not distributed thoroughly to the sperm cells. In contrary, we were using liposomal particle of lipids with size smaller than 100 nm, therefore it may due enhanced cellular absorption. It is an interesting observation but needed further study to clarify.

It is known fact that high body mass index (BMI) is correlated to various diseases including in changing certain hormones. Total sperm count have been found to be reduced and sperm cells abnormality in the person having higher BMI (Jensen et al., 2004). Another study shows sperm DNA and pregnancy outcome improves significantly by reducing abdominal fat (Faure et al., 2014). Total concentration of sperm cells is also found to be directly correlated with the intake of saturated fat (Jensen et al., 2013). All the articles suggested that optimal sperm quality somehow depend on body fats.

It is important to mention that the sperm used for current experiment were from different individuals. Therefore biological response may vary significantly as shown in *Figure 32*. Based on each donor's personal life and habitat, the sperm quality can behave differently. These data were preliminary and sperm donors were blinded.

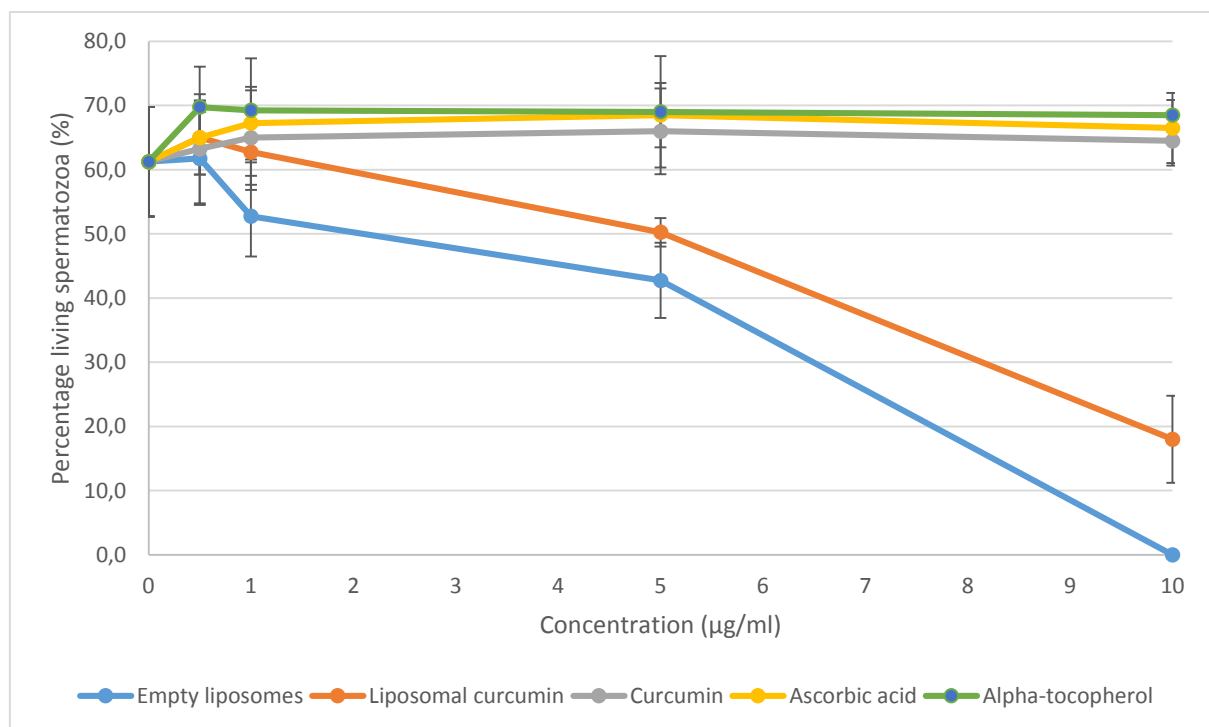
When working with sperm, it is important to always mention time after semen collection. The longer time the sperm suspension are at temperatures out of 37 °C range, the fewer living sperm cells will be found in the suspension. A big factor contributing to this is pH. A normal pH range is specified to be around 7.2. When the semen leave the male body, the pH increases with time. A pH outside the normal range is harmful to sperm (World Health, 2010). An evaluation of viability after 24 hours of incubation is therefore not a good enough estimation since the starting point will be between 2-4 hours.

However, the sperm used in this experiment were samples given from the IVF-clinic at the University Hospital of North Norway (UNN). The semen that was used had a good quality with 80 – 90 % alive sperm cells. With this in mind, it was assumed that the starting point of every suspension was the same even though more adjustment should have been done if this research will carry on.

In summary, these preliminary data showed that the viability of the sperm cells improved only at lower lipid concentrations. The same results were found for liposomal curcumin. It is difficult to confirm if the viability is increased by curcumin alone, lipid only or both. But it was seen that the percentage of viable sperm cells were significantly raised when the concentrations of lipid were reduced.

### 5.7.2 Method 2: Sperm viability

In this method, similar experiments were carried out as described previously. Instead of using swim up medium-HTF as method 1, it was used Protein Plus Fertilizing HTF medium. Application of liposomes and dilutions were carried out as mentioned in experimental Section 4.6.2.2. The effect of curcumin, vitamin C, vitamin E and liposomal curcumin on sperm motility and viability were observed manually under the light microscope. Empty liposome was taken as control for liposomal curcumin.



**Figure 33:** The effect of anti-oxidant solutions and liposomal curcumin on sperm viability *in vitro*.

As seen in *Figure 33*, curcumin, ascorbic acid and  $\alpha$ -tocopherol had the similar trend, while use of empty liposomes and liposomal curcumin decreased sperm cells viability. Liposomal formulation showed the increased viability on sperm cells comparing to empty liposome. T-test was used to determine if some of the anti-oxidants affected the viability of sperm cells significantly.

For the anti-oxidants that were used directly, had a relatively unchanged viability at concentrations up to 10  $\mu\text{g/ml}$  and was significantly different at a 95 to 99 % confidence level from the liposomal formulations as shown in *Figure 33*. The findings of vitamin C and vitamin E were as expected. For instance, lower ascorbic acid concentration is found in the seminal plasma of infertile men compared to fertile men, indicating it's important function for male fertility (Lewis et al.). Addition to that, ascorbic acid also improved the motility and viability of human sperm cells (Verma and Kanwar, 1998), and prevented sperm DNA damage (Hughes et al., 1998).

$\alpha$ -Tocopherol has also shown protective role on human sperm cells as found in different clinical studies. Most of them imply that  $\alpha$ -tocopherol can improve sperm viability, membrane integrity, motility and morphology (Agarwal et al., 2003, Lanzafame et al., 2009, Bansal and Bilaspuri, 2010).  $\alpha$ -Tocopherol has also a positive effect on the DNA oxidation (Hughes et al., 1998), and in a placebo controlled double-blind study, male with asthenozoospermia increased sperm motility after administrating  $\alpha$ -tocopherol 100 mg daily for 6 months, and the pregnancy rate was increased (Suleiman et al., 1996).

The influence of curcumin on sperm cells is not known so far. However, curcumin is found as a potent as vitamin C or vitamin E. Although, it is very preliminary report, it seems like the toxicity of the lipid can be reduced with the presence of curcumin. There were found significant differences at 95 % confidence level between empty liposomes and liposomal curcumin at 0.5  $\mu\text{g/ml}$  concentrations or higher as shown in *Figure 33*. The amount of lipid in the liposomal formulations had negative effect on the viability of sperm cells, while presence of anti-oxidant curcumin reduce that. It should be noted that the curcumin-lipid ratio was 1:10 (w/w). Further experiments are needed with protective effect of curcumin against lipid toxicity at higher concentration of curcumin.

The results obtained in the method 1 and method 2 are compared in *Table 9*. Some different results were observed.

**Table 9:** Comparing the effect of liposomal formulations in method 1 and method 2.

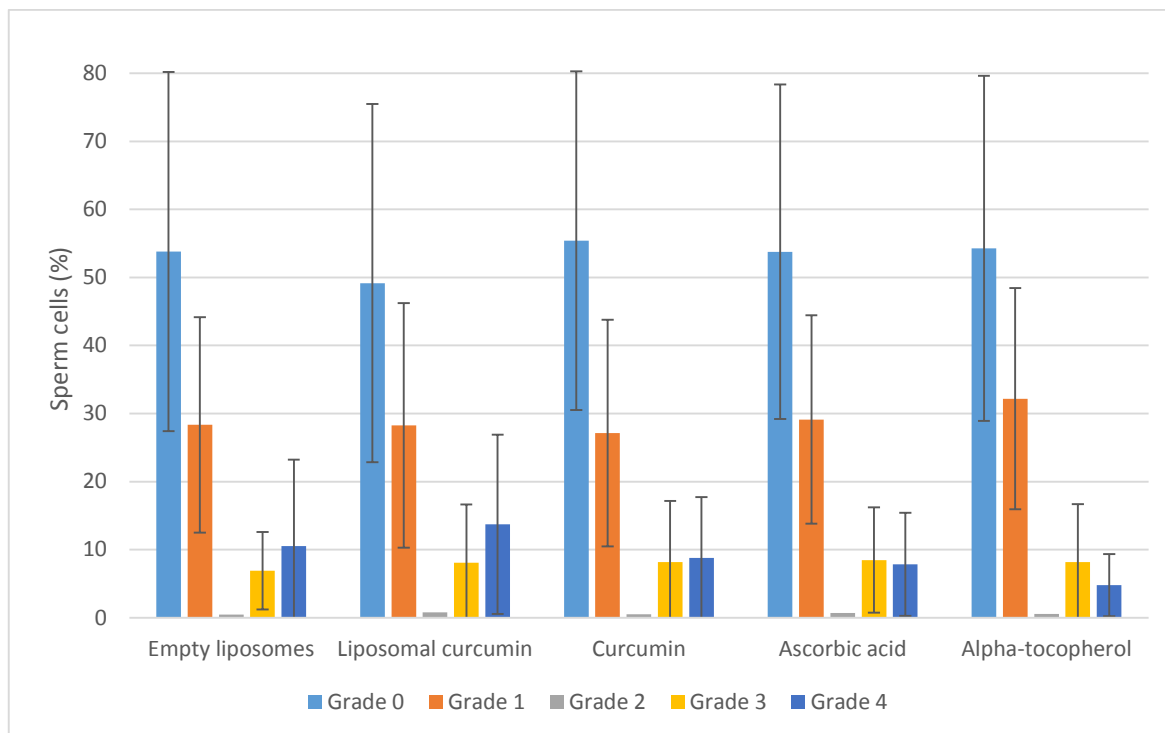
Lipid ( $\mu\text{g/ml}$ )	Method 1		Method 2	
	Empty liposomes	Liposomal curcumin	Empty liposomes	Liposomal curcumin
0	$70 \pm 0$	$60 \pm 0$	$61.3 \pm 8.5$	$61.3 \pm 8.5$
0,5	0	$0.4 \pm 1$	$61.8 \pm 7.2$	$65.0 \pm 5.8$
1	0	$0.1 \pm 0.4$	$52.8 \pm 6.3$	$62.8 \pm 5.9$
5	0	0	$42.8 \pm 5.9$	$50.3 \pm 2.2$
10	0	0	$0.0 \pm 0$	$18.0 \pm 6.8$

The values are presented as mean  $\pm$  SD ( $n \geq 2$ ) with two replicates.

Dilution methods and medium were completely different in these two methods. In addition, due to increased working efficiency, sperm cells were exposed time outside the incubator is minimized while working in method 2. All these factors might contributed for different results. However, similar trends were observed in both methods as lipid concentration increased the viability of the sperm cells decreased.

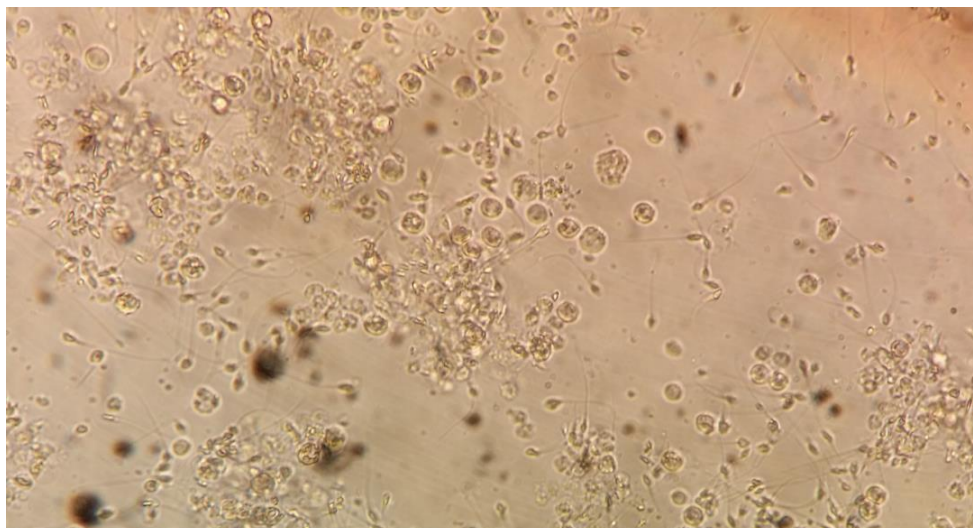
### 5.7.3 Sperm motility and viability

Concentration of the anti-oxidants were set as described previously in *Section 4.6.3*. The concentration of curcumin, ascorbic acid and  $\alpha$ -tocopherol were set to contain same molarity curcumin in liposomal curcumin (*Table 2*). The semen suspensions were prepared  $2.7 \pm 0.8$  hours after hand in from donor. The observations were done  $23 \pm 0.9$  hours after the treatment. During the observations, viability and motility grade were observed and results are presented as shown below in *Figure 34*.



**Figure 34:** The effect of anti-oxidant solutions and liposomal suspension on sperm motility and viability. The concentration of the lipid was 0.40  $\mu\text{g/ml}$  for empty liposomes and liposomal curcumin. Concentration of curcumin was 0.04  $\mu\text{g/ml}$ , 0.05  $\mu\text{g/ml}$  for ascorbic acid and 0.02  $\mu\text{g/ml}$  for  $\alpha$ -tocopherol.

The trend for all the tested liposomal formulations and anti-oxidants were very similar. Around 50 % of the sperm cells were dead. 30 % of the alive sperm cells were in motility grade 1, and the remaining 20 % were accounted for motility grade 3 and 4. For correct method of counting, one could apply computer assisted sperm analysis (CASA). One of the limitations is due to the manual counting. For instance, it was seen that the sperm cells had a tendency to agglutinate to each other after 24 hours of incubation as shown in *Figure 35*. Further improvement in methodology is essential.



**Figure 35:** Sperm agglutination observed in light microscope (x200).

The large variation as seen by the big standard deviation clearly show that the number of samples has to be increased.

In addition, it was used fresh semen directly without purification in this experiment. All ejaculates have a proportion of leukocytes, majorly of macrophages and neutrophils in the seminal plasma. They have high production of ROS which is not favourable on sperm functionalities (Bansal and Bilaspuri, 2010, Chen et al., 2013, Agarwal et al., 2014). Since the leukocytes were not removed in this experiment it may affect the results. Purified semen sample surely gave the better results.

In summary, it was expected that at a concentration of 0.4  $\mu\text{g/ml}$  lipid would give around 10 % viability because of *Section 5.7.1*. However, it was found around 50 % dead sperm cells, 30 % alive sperm cells in motility grade 1 and around 20 % were accounted for motility grade 3 and 4. The trend for all the anti-oxidant solutions and liposomal suspensions were very similar. No significant differences were found between all of them (with or without liposomal formulation). Further studies are needed to validate the findings in this experiment.



## 6 CONCLUSION

The present research work was mainly focused on the effect of anti-oxidants on sperm cells motility and viability *in vitro*. A clinically potential anti-oxidant, curcumin was chosen because of its multi-targeted anti-oxidative and anti-inflammatory activities as well as high safety profile. However, because of poor solubility it could not be used directly. Therefore by the application of nanotechnology, liposomes incorporated with curcumin was prepared and optimized for such purpose. A method was developed for the preparation of liposomes with curcumin with the high size uniformity and high entrapment efficiency (>80 %). With extrusion method, the liposomes were found very monodispersed and uniform in size which was maintained for 5 months period of storage.

Anti-oxidative activities of curcumin were examined with the radical scavenging capacity by ABTS and DPPH assay methods. The EC<sub>50</sub> for curcumin were 5.82  $\mu$ M for ABTS<sup>•+</sup> solution and 9.13  $\mu$ M for DPPH<sup>•</sup> solution, respectively. This result clearly showed that curcumin is as potent as widely discussed anti-oxidants such as vitamin C or vitamin E. All tested anti-oxidants namely curcumin, vitamin C and vitamin E improved the motility and viability of the sperm in this preliminary pilot study.

Another interesting finding in this study was that liposomal formulations had negative effect on sperm motility comparing to curcumin. Empty liposomes were taken as the control for the liposomal curcumin and showed high level of toxicity to sperm cells at the concentration of 0.5  $\mu$ g/ml. Comparing to empty liposome, liposomal curcumin showed an increased motility at the same lipid concentration. This study clearly indicated a strong toxicity to the sperm cells by phosphatidylcholine at the concentrations more than 0.5  $\mu$ g/ml. It was not clear whether such toxicity is due to particular lipid. Further studies are needed to explore and understand fully the toxicity of lipid on the sperm cells.



## 7 PERSPECTIVES

The results found in this thesis are preliminary pilot study. Current study was the first of its kind and mainly focused on the experimental method developments. Curcumin seemed to enhance the motility and viability of human sperm cells *in vitro* probably due to its anti-oxidative effect. Further studies on curcumin on larger scale with the formulation including other types of lipids are also necessary. It could also be interesting to see if the morphology of the sperm cells changes, not only observation of motility and viability.

Liposomal formulation in certain concentrations had an impact on the viability of sperm cells. Taking in consideration that phosphatidylcholine was the dominant component in liposomes, this was an unexpected finding. **Does this mean that soluble fat can affect the fertility?** A focused study is necessary in order to link obesity and infertility.

It would be interesting to see the effect of liposomes with other types of lipids. One should note that 44 % of the phospholipids found in seminal plasma contains sphingomyelin, while phosphatidylethanolamine contributes as the second most abundant lipid in seminal plasma and human sperm cells (Poulos and White, 1973). The literature states that phosphatidylethanolamine is drastically reduced in infertile men, while phosphatidylserine is significantly increased (Gulaya et al., 2001). If phosphatidylethanolamine is so important for fertility as the literature report, maybe a liposomal formulation with this phospholipid can open a new way of transporting reproductive at least by improving human semen quality.

It is important to remember that this was an *in vitro* study, which was more relevant for ART. *In vivo*, the human body has a lot of physiological barriers like blood vessel wall, extracellular matrix and interstitial fluid pressure gradients, among many others. Nanoparticles/liposomes have to overcome these barriers before they can act on the target cells.

Nanomedicine has shown a wide range of possibilities in diagnosis, treatment and prevention of diseases. Nanomedicine also reduces unwanted side effects. In spite of several possibilities and difficulties, nanotechnology is still evolving. With this in mind, a lot of ethical questions are raised whether it can have an impact on the normal evolution on humans.

I would like to end this by reminding that nanomedicine gives unlimited opportunities for the future, but also with a lot of considerations.



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