AMERICAN UNIVERSITY OF BEIRUT

MODULATION OF PHOSPHOLIPID MEMBRANES BY CURCUMIN-CONJUGATED SILVER NANOPARTICLES AND THEIR APPLICATION

by
NADINE WEHBE WEHBE

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Biology
of the Faculty of Arts and Sciences
at the American University of Beirut

Beirut, Lebanon
May 2018

AMERICAN UNIVERSITY OF BEIRUT

MODULATION OF PHOSPHOLIPID MEMBRANES BY SILVER NANOPARTICLES AND THEIR APPLICATION

by

NADINE WEHBE WEHBE

Approved by:

Dr. Elias Baydoun, Professor Biology	Advisor
Dr. Digambara Patra, Associate Professor Chemistry	Co-advisor
Dr. Roula Abdel-Massih, Professor Biology	Member of Committee

Date of thesis defense: May 2, 2018

AMERICAN UNIVERSITY OF BEIRUT

THESIS, DISSERTATION, PROJECT RELEASE FORM

Student Name: Wehbe (Last) Nadine (First) Wehbe (Middle)

● Master's Thesis ○ Master's Project ○ Doctoral Dissertation

☑ I authorize the American University of Beirut to: (a) reproduce hard or electronic copies of my thesis, dissertation, or project; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes.

☐ I authorize the American University of Beirut, to: (a) reproduce hard or electronic copies of it; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes
after :**One----year from the date of submission of my thesis, dissertation, or project.**
Two----years from the date of submission of my thesis, dissertation, or project.
Three----years from the date of submission of my thesis, dissertation, or project.

Nadine Wehbe May 11, 2018

Signature Date

ACKNOWLEDGMENTS

Firstly, I would like to thank my advisor, Dr. Elias Baydoun, for his generous help and support. I would also like to thank Dr. Digambara Patra and Dr. Roula Abdel-massih for their support and kind advice. In addition, I would like to thank my dear friends for making this journey a memorable one. Last but not least, a special thanks goes to my family for all their encouragement and prayers.

AN ABSTRACT OF THE THESIS OF

Nadine Wehbe Wehbe for Master of Science
Major: Biology

Title: Modulation of Phospholipid Membranes by Curcumin-conjugated Silver Nanoparticles and their Application

Nanotechnology is a field of science involving the synthesis and development of nanomaterials. Silver nanoparticles (AgNPs) have been incorporated in several biomedical and environmental applications because of their chemical stability, catalytic activity, and enhanced conductivity. Green synthetic routes have been an emerging field to synthesize AgNPs. These routes help minimize the use of hazardous substances therefore reducing the formation of toxic products. Curcumin can be used as a reducing agent in the green synthesis of AgNPs. Curcumin is the active ingredient of turmeric, a medicinal and dietary spice extracted from *Curcuma longa*. Although extensive research has proven curcumin as a potential drug for various diseases such as Alzheimer's disease, carcinogenesis, and inflammatory disorders, curcumin's poor bioavailability limits its therapeutic efficacy. Therefore, numerous approaches have been developed to enhance curcumin's therapeutic utility. These include co-administration of curcumin and adjuvants or encapsulation of curcumin within the membrane of delivery systems such as liposomes. The impact of AgNPs on phospholipid membrane properties is not clearly understood yet. By applying curcumin as a probe molecule, this work was done to investigate the effect of AgNPs on membrane properties such as permeability and phase transition temperature using 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) liposomes as a model for phospholipids membranes. In addition, cytotoxicity of curcumin and curcumin-AgNPs (cur-AgNPs) was assessed against Human cervical cancer (HeLa) cells. We concluded that AgNPs at low concentration decrease the partition of curcumin into DMPC liposomes by ~ 4-fold. In the presence of AgNPs, curcumin was found to be located close to the stern layer of DMPC liposomes by using a hydrophobic quencher, cetylpyridinium bromide (CPB). In addition, AgNPs broadened the phase transition temperature of DMPC liposomes as it ranged from 20°C to 35°C. Low concentrations of AgNPs were found to have cytotoxic effect against HeLa cells, and a synergistic effect was detected when combining AgNPs and curcumin.

CONTENTS

ILLUSTRATIONS

TABLES

ABBREVIATIONS

Ag NPs	Silver nanoparticles
AP-1	Activator protein-1
CAMs	Cell adhesion molecules
CDK4	Cyclin dependent kinase 4
CDK6	Cyclin dependent kinase 6
CPB	Cetylpyridinium bromide
COX-I	Cyclooxygenase-I
COX-II	Cyclooxygenase-II
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DMSO	Dimethylsulfoxide
HeLa cells	Human cervical cancer cells
HPV	Human papilloma virus
Kp	Partition Coefficient
K_{SV}	Stern-Volmer constant
LSPR	Localized Surface Plasmon Resonance
LUVs	Large unilamellar vesicles
MLVs	Large multilamellar vesicles
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa-B
PC	Phosphatidylcholine

PE	Phosphatidylethanolamine
PS	Phosphatidylserine
ROS	Reactive oxygen species
SEM	Scanning electron microscope
SDS	Sodium dodecyl sulfate
SUVs	Small unilamellar vesicles

CHAPTER I

INTRODUCTION

Green chemistry is an emerging field used in the synthesis of nanoparticles. It is based on the use of eco-friendly and nontoxic agents to help reduce the formation of toxic products. Recently, curcumin has been used as a reducing and stabilizing agent to synthesize AgNPs via green synthetic routes (Shameli *et al.,* 2012). Curcumin is the active ingredient in turmeric, a gold-colored spice extracted from the plant *Curcuma longa*. Curcumin is widely known for its therapeutic use as an antibacterial, anti-inflammatory, and anticancer agent. It is also known to enhance wound healing, inhibit platelet aggregation, reduce blood cholesterol, and suppress symptoms related to Alzheimer's disease (Gupta *et al.,* 2012). Altogether, these beneficial uses contributed to establishing curcumin as a dietary supplement with a safety dosage of up to 8g/day (Chainani-Wu 2003).

In the past decades, the use of AgNPs has dramatically increased because of their biological applications such as antibacterial and anticancer agents, drug delivery, and diagnosis. AgNPs are known to have distinctive properties including chemical stability, catalytic activity, enhanced conductivity, and optical functions (Wei *et al.,* 2015). AgNPs are known to be toxic to human hepatocytes, fibroblasts, and glioblastomas. They also have anticancer effects against breast, leukemia, liver, and lung cancer cells (Riaz *et al.,* 2017). However, the impact of AgNPs on membrane properties is not clearly understood yet.

Previously in our lab, the partition coefficient of curcumin with DMPC liposomes

was measured and found to be high. It was concluded that curcumin binds strongly to liposomes, and at high concentration it can influence the phase transition temperature of liposomes. However, at a low concentration curcumin does not influence it significantly (El Khoury and Patra 2013). Thus, curcumin at a low concentration range can be used as a probe to investigate the effect of AgNPs on the properties of phospholipid membranes.

The aim of this work is to

1. Synthesize curcumin-conjugated AgNPs.
2. Study the effect of AgNPs on the partition of curcumin into DMPC liposomes.
3. Determine the effect of AgNPs on the fluorescence quenching of curcumin by CPB in DMPC liposomes.
4. Examine the effect of AgNPs on the phase transition temperature of DMPC liposomes.
5. Assess anti-proliferative effect of curcumin and cur-AgNPs against HeLa cells.

CHAPTER II

LITERATURE REVIEW

A. Nanoparticles

Nanotechnology is a field of science involving the synthesis and development of nanomaterials, which are usually 1 to 100 nm in size. Metallic nanoparticles, made up of metal precursors, have shown to exhibit unique physical and chemical properties compared to their bulk counterparts. Therefore, they have been incorporated in several biological applications such as antibacterial and antifungal agents, drug targeting and delivery, wound dressing, and disinfectants (Wei *et al.,* 2015). Noble metals such as gold, palladium, platinum, and silver are mostly used for the preparation of metallic nanoparticles.

1. General Properties of Metallic Nanoparticles

As mentioned earlier, metallic nanoparticles have unique physicochemical properties. These include optical, magnetic, and mechanical properties among several others.

Noble metal nanoparticles exhibit a strong UV-visible extinction band, which is not found in the spectrum of their bulk counterparts, known as the localized surface plasmon resonance (LSPR). LSPR is an optical phenomenon where an electromagnetic wave is trapped on the metal surface. This phenomenon is generated when the incident electromagnetic field interacts with the metal nanoparticle. The electrons on the metal's surface oscillate collectively and coherently in resonance with the light frequency creating

LSPR on the metal nanoparticle surface (Fong and Yung 2013). The peak wavelength of the LSPR spectrum depends on the size and shape of the nanoparticles as well as the properties of its surrounding environment. Noble metal nanoparticles are implicated in sensing and imaging techniques due to their optical properties (Eustis and El-Sayed 2006).

Magnetic nanoparticles are made up of a magnetic material and a functional chemical material. Having magnetic properties, these nanoparticles obey Coulomb's law: same charges rebel and opposite charges attract. Therefore, they can be manipulated by an external magnetic field. Magnetic nanoparticles are widely implicated in biomedicine since they can be used for tissue targeting and drug delivery. These nanoparticles can then be easily manipulated by a magnetic field, which is permeable to human tissues (Pankhurst *et al.,* 2003).

Mechanical properties of nanoparticles consist of several parameters such as elasticity, hardness, coagulation, and surface coating. These properties enhance the use of metal nanoparticles in coatings, nanomanufacturing, surface engineering, and tribology (Guo *et al.,* 2014).

2. Silver Nanoparticles

Popularity of silver is due to the fact that it is more affordable than other noble metals such as gold, palladium, and platinum. In addition, AgNPs are commonly used because of their distinctive properties such as chemical stability, catalytic activity, enhanced conductivity, and optical functions (Wei *et al.,* 2015). Synthesis methods of AgNPs along with their diverse applications and potential toxicity are discussed in the following sections.

a. Synthesis Methods of AgNPs

Synthesis methods of AgNPs can be categorized as chemical, physical and biological methods. Synthesis of AgNPs in solution via chemical methods, such as chemical reduction process (Landage *et al.,* 2014), electrochemical techniques (Roldán *et al.,* 2013), and pyrolysis (Sotiriou *et al.,* 2011), contains three main components: metal precursors, reducing agents, and stabilizing agents. Synthesis via physical methods has some advantages, including absence of toxic chemicals, fast processing time, and narrow size distribution of formed AgNPs (Asanithi *et al.,* 2012). Examples of physical methods are arc-discharge method (Tien *et al.,* 2008), direct current magnetron sputtering (Asanithi *et al.,* 2012), and physical vapor condensation (Abou El-Nour *et al.,* 2010). In biological methods, nontoxic molecules such as antioxidants, carbohydrates, and proteins, are used as reducing agents and stabilizers. Microorganisms, including bacteria (Shivaji *et al.,* 2011), fungi (G. Li *et al.,* 2012), and yeasts (Mourato *et al.,* 2011) help produce those nontoxic molecules.

The most popular method used to synthesize AgNPs as stable colloidal dispersions is the chemical reduction of silver salts (Abou El-Nour *et al.,* 2010). Some widely used reducing agents are alcohol, ascorbic acid, sodium borohydride, and sodium citrate (Wei *et al.,* 2015). The reduction of silver ions (Ag+) in aqueous solution leads to the formation of silver atoms (Ag0). This is followed by agglomeration resulting in the formation of colloidal silver nanoparticles. It is important to stabilize the dispersive nanoparticles during their synthesis. Examples of stabilizing/capping agents used for this matter are polyvinylpyrrolidone, starch, and sodium carboxyl methylcellulose (Landage *et al.,* 2014).

Since chemical reduction involves the use of hazardous chemicals, it is considered harmful and should be replaced with a safer method. Green synthetic routes have been an emerging field to synthesize AgNPs (El Khoury *et al.,* 2015). These routes help minimize the use of hazardous substances therefore reducing the formation of toxic products. To be considered as a green chemistry route, the used method should be evaluated based on the selection of solvent medium, eco-friendly reducing agent, and nontoxic stabilizing/capping agents.

b. Applications of AgNPs

In the past decades, the use of AgNPs has dramatically increased because of their therapeutic applications. AgNPs function as antifungal, antibacterial, and anticancer agents. They are also used in drug delivery and diagnosis (Wei *et al.,* 2015). One study showed that AgNPs have an inhibitory effect against the growth of *Trichosporon asahii,* an emerging fungal pathogen. Based on electron microscopy observations, AgNPs inhibited growth of *T.asahii* by damaging the fungal cell wall and cellular organs (Xia *et al.,* 2016). In a recent study, antibacterial and antifungal activities of AgNPs were examined. AgNPs (30 g filled in a disc) had good antibacterial activity against tested strains of *Escherichia coli*, *Proteus vulgaris, Staphylococcus aureus, Staphylococcus epidermidis,* and *Vibro cholera.* Inhibition zones were observed as 16 ± 0.34, 14 ± 0.03, 12 ± 0.24, 14 ± 0.94, and 12 ± 0.26 mM, respectively. AgNPs were also found to have a concentration- and time-dependent anticancer effect against MCF-7 breast cancer cells. IC_{50} was found to be 70 g/ml and 50 g/ml after 24-hour and 48-hour incubation, respectively (Majeed *et al.,* 2016).

In addition, AgNPs have been used in many manufacturing processes and end products. They are found in cosmetics, home appliances, and textiles as well as incorporated in medical devices such as pacemakers and wound dressings. AgNPs can be used in their liquid form, such as in coating/spray agents and shampoo, or in solid form such as in soap (Wei *et al.,* 2015). Wide and increasing use of AgNPs in biomedical and environmental applications necessitates understanding the effect of AgNPs on human health, particularly their toxicity.

c. AgNPs and their Toxicity

Toxicity of AgNPs has been widely studied in numerous cell types. AgNPs are found to be toxic to human hepatocytes, fibroblasts and glioblastomas. They also have anticancer effects against breast, leukemia, liver, and lung cancer cells (Majeed et al., 2016). AgNPs mediate their cytotoxicity primarily by oxidative stress due to the release of silver ions from their surface (Zhang et al.,2014). For most cells, the uptake of AgNPs occurs mainly through endosomal or lysosomal endocytosis (Wei et al.,2015). Silver ions target organelles such as the mitochondria and the nucleus. By interacting with membrane proteins, silver ions cause DNA damage, cell morphology alteration, inflammation, and mitochondrial dysfunction leading to cell death by apoptosis or necrosis (Asharaniet al.,2012; Zhang et al.,2014).

The size, shape, and surface chemistry of AgNPs influence their cytotoxicity. Several studies have shown that smaller sized AgNPs exhibit greater cytotoxicity due their enhanced ability to pass through the cell membrane resulting in a greater cellular uptake

(W. Liu *et al.,* 2010; Gliga *et al.,* 2014). A study investigated the cytotoxicity of silver nanowires, spherical AgNPs, and silver microparticles on lung epithelial cells. Results showed that the highest level of cytotoxicity was exhibited by silver nanowires. This was thought to be due to the direct interactions of silver nanowires with the cell membrane rather their cellular uptake by endocytosis as in the case of spherical AgNPs (Stoehr *et al.,* 2011). In addition to their size and shape, surface chemistry of AgNPs was shown to affect the cellular uptake of AgNPs and thus their degree of cytotoxicity (Zhang *et al.,* 2014). Higher toxicity is generally associated with positively charged AgNPs compared to negatively charged or neutral AgNPs. This could be due to their higher affinity to the negatively charged plasma membranes (Suresh *et al.,* 2012). Up to date, the influence of AgNPs on human health is controversial. More studies are required to further understand the safety and toxicity of AgNPs.

B. Curcumin

Curcuma longa is a perennial herb that belongs to the ginger family, Zingiberaceae, and is widely cultivated in tropical regions of Asia. The plant's rhizome, turmeric, is a spice of golden color used as food additive, preservative, and coloring agent. India is the primary exporter of turmeric, which is known as the "Indian Saffron" in Europe. Turmeric has been known for its therapeutic activities since the time of Ayurveda (1900 BC), the science of long life (Aggarwal *et al.,* 2007). Traditional medicine uses turmeric as an herb for arthritis, conjunctivitis, chicken pox, skin cancer, and wound healing (Nasri *et al.,* 2014).

Turmeric contains a variety of phytochemicals, one of which is curcumin. Curcumin is the phytochemical that gives turmeric its yellow color, and it is responsible for most of turmeric's therapeutic effects. Curcumin is estimated to constitute 2-5% of turmeric (Aggarwal *et al.,* 2015). Vogel and Pelletier first isolated curcumin in 1815 (Vogel and Pelletier 1815). However, its chemical structure was not identified until 1910 by Milobedzka and Lampe as (1E, 6E)-1,7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione or diferuloylmethane (Milobedzka *et al.,* 1910) as shown in Figure 1.

Curcumin is a hydrophobic polyphenol with a vibrant yellow color allowing it to be used as a dye. It is poorly soluble in aqueous media, but it is soluble in organic solvents such as acetone, dimethylsulfoxide (DMSO), ethanol, and methanol. Curcumin's molecular formula is $C_{21}H_{20}O_6$, and it has a molecular weight of 368.37 g/mol and a melting point of 183°C (Goel *et al.,* 2008).

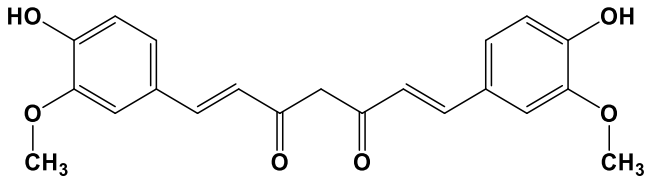


Figure 1: Chemical structure of curcumin.

1. Therapeutic Uses of Curcumin

Curcumin is widely known as a therapeutic agent having a wide variety of biological uses, including antibacterial, anti-inflammatory, antioxidant, and anticancer. In addition, curcumin is known to enhance wound healing, inhibit platelet aggregation, reduce blood cholesterol, suppress thrombosis and myocardial infarction, and suppress symptoms

related to Alzheimer's disease (Gupta *et al.,* 2012). Altogether, these beneficial uses contributed to establishing curcumin as a dietary supplement with a safety dosage of up to 8g/day (Chainani-Wu 2003).

a. Antibacterial Activity

Public health has been at risk of antimicrobial resistance since the 20th century. Therefore, despite the progress in the industry in developing effective antibacterial drugs, there is always a need for new drugs due to the development of multidrug resistant bacteria (Wise *et al.,* 1998). Extensive research has proven curcumin to be a suitable agent against a wide variety of bacteria. A recent study revealed antibacterial activity of curcumin against standard bacterial strains, such as *Bacillus subtilis*, *Escherichia coli*, *Enterococcus faecalis*, and *Klebsiella pneumonia* at high concentrations. The minimum inhibitory concentration was found to be in the range between 129 μg/ml and 293 μg/ml (Gunes *et al.,* 2016). Another study showed a strong concentration- and time-dependent antibacterial activity of curcumin against Gram-positive bacterial strains, *Staphylococcus aureus* and *Enterococcus faecalis*, and Gram-negative bacterial strains, *Escherichia coli* and *Pseudomonas aeruginosa*. A concentration of 100 μM was able to kill 100% of the bacteria after a 2-hour exposure. It was shown that curcumin mediated its killing effect by damaging the cell membrane (Tyagi *et al.,* 2015).

b. Anti-inflammatory and Antioxidant Activities

Oxidative stress and inflammation have similar pathological processes. In fact, they can easily induce one another. For example, it is known that inflammatory cells liberate a

number of reactive species at the inflammation site leading to oxidative stress (Biswas 2016). Studies have proven curcumin as an anti-inflammatory and antioxidant agent. Curcumin was shown to exhibit a stronger antioxidant activity compared to vitamins C and E (Toda *et al.,* 1985). It was found to be an effective scavenger of a number of reactive oxygen species (ROS) including hydroxyl radicals and superoxide anions (Reddy and Lokesh 1994). A study investigated the anti-inflammatory activity of curcumin against cyclooxygenase-I (COX-I) and cyclooxygenase-II (COX-II) enzymes and its antioxidant activity against liposome peroxidation initiated by FeCl2 solution. It was shown that curcumin, at a concentration of 125 μg/ml, inhibited COX-I enzyme by 32% and COX-II enzyme by 89.7%, and 100 μg/ml of curcumin was able to inhibit liposome peroxidation by 58% (Ramsewak *et al.,* 2000).

c. Anticancer Activity

According to the Center for Disease Control and Prevention, 14.1 million new cancer cases have been diagnosed in 2012. It is expected to increase to 19.3 million by 2025 (Ries *et al.,* 2007). Cancer is a complex disease characterized by the accumulation of gene mutations followed by abnormal cell proliferation and growth. It is marked by metastasis into the body's vital organs through invasion and angiogenesis. In all types of cancer, inflammation is a major factor of initiation and progression. Since it is known for both its anti-inflammatory and anticancer activity, curcumin has been shown to have a wide range of therapeutic effects in many cancers. Curcumin is well known for its anticancer activity because it has the ability to suppress the growth of a variety of tumor cell lines by modulating the activity of several molecular factors, which are implicated in

carcinogenesis. It also has the ability to control the expression of genes involved in angiogenesis, cell proliferation, and metastasis (Shishodia *et al.,* 2007).

i. Types of Cancer

Curcumin has a multi-targeting ability against a number of molecular factors involved in carcinogenesis. This is the key to why curcumin has antitumor effects against various cancers. Several preclinical and clinical studies have proven curcumin to have antitumor effects against many cancers including breast, cervical, hepatic, lung, pancreatic, and intestinal.

Breast cancer is most commonly and frequently diagnosed at an average age of 61 years in women (Ries *et al.,* 2007). Although therapeutic interventions have enhanced the survival rate of breast cancer patients, more than 60% of deaths from breast cancer are due to late recurrences (Widakowich *et al.,* 2007). A study established that curcumin has an anti-proliferative effect against MDA-MB-231 and BT-483 breast cancer cells. Curcumin mediates its effect by inhibiting the nuclear factor kappa-B (NF-κB) and cyclin D1 transcription (Liu *et al.,* 2009). In a human breast cancer xenograft model, dietary administration of curcumin suppressed NF-κB pathway and inhibited the incidence of breast cancer metastasis to the lung (Aggarwal *et al.,* 2005).

Cervical cancer is the most predominant cancer in women in several developing countries. It is usually diagnosed in young patients at the age of 48 years (Ries *et al.,* 2007). A study on HPV-infected cells showed that curcumin is cytotoxic to cervical cancer cells by down-regulating NF-κB and Activator protein-1 (AP-1) expression levels and inhibiting the expression of viral oncogenes E6 and E7 (Divya and Pillai 2006). In cervical cancer

xenograft mice models, oral administration of 1,000 or 1,500 mg/kg of curcumin daily for one month caused tumor growth reduction by 21.03% and 35.57%, respectively. Curcumin inhibited tumor growth and angiogenesis by down-regulating COX-2 and VEGF expression (Yoysungnoen-Chintana *et al.,* 2014).

Pancreatic cancer is one of the most common cancers, and it accounts for 6% of all cancer-related deaths in both men and women (Ries *et al.,* 2007). Despite therapeutic advances in the treatment and prevention, patients with pancreatic cancer have around 5-year survival of 5% (Jemal *et al.,* 2007). Curcumin was shown to inhibit tumor growth in pancreatic cancer cells by suppressing NF-κB expression and growth control molecules induced by it (Li *et al.,* 2004). In a phase II clinical trial, patients with advanced pancreatic cancer received 8g curcumin daily for 8 weeks. Although it has poor oral bioavailability, curcumin down-regulated the expression of NF-κB. It also showed antitumor activity in two patients, one of which had 73% tumor decrease. Curcumin was found to be tolerable with no toxicity for 18 months (Dhillon *et al.,* 2008).

ii. Molecular Targets of Curcumin

Curcumin has been shown to target several molecules involved in carcinogenesis, and it has the ability to modulate the expression of transcription factors, inflammatory enzymes, protein kinases, and adhesion molecules among many others.

Activation of NF-κB is a common feature of most cancers and acts by suppressing apoptosis in many cancer cells. It is also implicated in chemoresistance (Wang *et al.,* 1996). Curcumin was shown to suppress the activation of NF-κB induced by tumor promoters such as hydrogen peroxide (Singh and Aggarwal 1995). A study showed that curcumin

induced apoptosis in human melanoma cells by inhibiting NF-κB activation (Philip and Kundu 2003). Curcumin suppresses NF-κB activation through the impairment of the ubiquitin-proteasome system, which is the cell's principle mechanism for protein degradation. The inhibition of proteasome activity leads to an increase in the half-life of IκB-α, which is a substrate of the proteasome. IκB-α keeps NF-κB in its inactive state in the cytoplasm and ultimately down-regulates the activation of NF-κB (Dikshit *et al.,* 2006).

AP-1 is a family of transcription factors that has been linked with the proliferation and transformation of tumor cells. AP-1 is associated with the activation of NF-κB (Karin *et al.,* 1997). Curcumin suppresses the activation of the stress-activated kinase Jun N-terminal Kinase (JNK) (Chen and Tan 1998). JNK phosphorylates c-Jun resulting in the activation of AP-1(Xia *et al.,* 2000). AP-1 is also known to play a crucial role in the regulation of high-risk human papilloma viruses (HPVs), which are associated with the development of cervical cancer. A study revealed that curcumin has the ability to down-regulate AP-1 activity in HeLa cells, leading to the suppression of HPV oncogene expression (Prusty and Das 2005).

Cyclin D1 is an important regulator of cell progression through the first gap phase of the cell cycle. Cyclin D1 forms an active complex by binding to cyclin dependent kinases 4 and 6 (CDK4 and CDK6). This active complex promotes cell cycle progression by phosphorylating and inactivating retinoblastoma protein (pRb), a nuclear phosphoprotein that exerts growth-inhibitory effects (Giacinti and Giordano 2006). The loss of pRb regulation is the hallmark of cancer. Overexpression of Cyclin D1 is linked to the development of various cancers (Alao 2007). Studies have shown curcumin to down-regulate the expression of cyclin D1 by blocking its interaction with CDK4/CDK6

therefore inhibiting the phosphorylation and inactivation of pRb. This may contribute to the anti-proliferative activity of curcumin against several tumor types (Mukhopadhyay *et al.,* 2002). It is also known that Cyclin D1 expression is regulated by NF-κB; therefore, curcumin's suppression of NF-κB activity leads to the down-regulation of cyclin D1 in multiple myeloma cells (Bharti *et al.,* 2003).

Cell adhesion molecules (CAMs), such as intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), integrins, and cadherins, are transmembrane proteins required for cell-extracellular matrix or cell-cell interactions. Alteration in the expression of CAMs may serve as a mean for the development and metastasis of tumor cells (Okegawa *et al.,* 2004). NF-κB is known to regulate the expression of these molecules. Therefore, down-regulation of NF-κB activation by curcumin helps mediate the down-regulation of CAMs. Curcumin was shown to block the cell surface expression of adhesion molecules in endothelial cells leading to a suppression of tumor cell adhesion to endothelial cells (Kumar *et al.,* 1998).

2. Curcumin: Limitations and Improvement

Although extensive research has proven curcumin as a good therapeutic agent against a number of health conditions, curcumin's poor bioavailability limits its therapeutic efficacy. Since curcumin has low solubility in aqueous media, its preparation and administration is very crucial. Curcumin is rapidly metabolized and eliminated from the body after administration resulting in its poor bioavailability (Anand *et al.,* 2007).

A pharmacokinetics study showed that after intravenous administration of 10 mg/kg curcumin in rats, a maximum concentration of 0.36 ± 0.05 μg/mL was found in the serum.

However, after an oral administration of 500 mg/kg curcumin, a maximum plasma concentration of 0.06 ± 0.01 μg/mL was reached. The oral bioavailability of curcumin was estimated to be ~ 1% (Yang *et al.,* 2007). In a human clinical trial, an oral dose of 3.6 g of curcumin resulted in a plasma concentration of 11.1 ± 0.6 nmol/L after an hour of administration (Sharma *et al.,* 2004). In order to overcome the limitations of curcumin, a number of approaches has been established which helped to enhance its solubility and bioavailability.

Co-administration of curcumin and adjuvants helps to block the metabolic processing of curcumin. One study showed that co-administration of curcumin and piperine, an inhibitor of intestinal and hepatic glucuronidase, was able to increase the bioavailability of curcumin by 2000% (Shoba *et al.,* 1998).

Another approach to enhance the bioavailability of curcumin is the encapsulation of curcumin within the membrane of delivery systems. These systems also help to deliver curcumin to its desired site for therapeutic applications. Liposomes, exosomes, and polymeric micelles are examples of delivery systems used to overcome curcumin's poor bioavailability.

Liposomes are synthetic vesicles similar to the phospholipid bilayer of a cell membrane. They have the ability to carry both hydrophilic and lipophilic drugs (Li *et al.,* 2014). Liposomal curcumin was shown to enhance the stability, bioavailability, and anticancer efficacy of curcumin. An *in vivo* study revealed that liposomal curcumin enhanced the bioavailability of curcumin when orally administered by 4.96-fold. This indicated a higher gastrointestinal absorption rate compared to free curcumin (Takahashi *et al.,* 2009).

Exosomes are naturally occurring small extracellular vesicles. They are excreted by cells upon the fusion of the multivascular body (MVB), an intermediate endocytic compartment, with the plasma membrane (Edgar 2016). A recent study evaluated the bioavailability of exosomal curcumin versus free curcumin in the lung, liver, and brain of mice. A concentration of 2.5 mg/kg of exosomal curcumin or free curcumin was administered. Exosomal curcumin increased the bioavailability of curcumin by 4- to 6-fold probably due to higher uptake, prolonged circulation, and protection from rapid hepatic degradation (Aqil *et al.,* 2017).

Polymeric micelles are self-assembled nanostructure composed of amphiphilic macromolecules formed in an aqueous solution (Xu *et al.,* 2013). In a human trial, the bioavailability of free curcumin and micellar curcumin was examined. An 88-fold increase in the bioavailability of curcumin was achieved after the oral administration of micellar curcumin compared to free curcumin (Kocher *et al.,* 2015).

C. Liposomes

Lipids have the ability to self-assemble in aqueous media and form bilayers, micelles, and liposomes as indicated in Figure 2. Liposomes are synthetic vesicles prepared to resemble the structure of a cell membrane. They are made up mainly of phospholipids, molecules having a hydrophilic head and a hydrophobic tail (Li *et al.,* 2014). Bangham and Horne (1964) were the first to show that liposomes spontaneously form when phospholipids are dispersed in an aqueous medium (Bangham and Horne 1964).

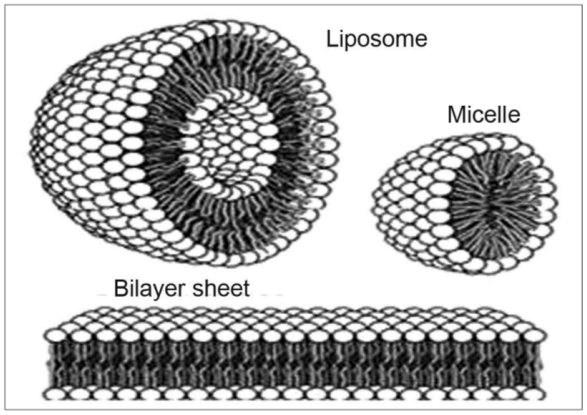


Figure 2: Schematic representation of a bilayer sheet, a micelle, and a liposome. (Tiwari et al., 2012)

1. Composition of Liposomes

Liposomes are made up of phospholipids, which consist of a hydrophilic head group and hydrophobic acyl chains both linked to an alcohol moiety. A variety of phospholipids exist due to variations in these groups and due to the source of phospholipids; this gives rise to a number of different liposomes. According to their alcohol, phospholipids can be either glycerophospholipids or sphingomyelins, having glycerol or sphingosine as their backbone, respectively.

Glycerophospholipids are the main phospholipids in eukaryotic cells. Their chemical structures can be classified based on several variations such as the head group and the length of nonpolar moiety. Based on the head group, glycerophospholipids can be grouped as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) among many others. Variations in the length of the nonpolar moiety lead to glycerophospholipids such as dimyristoyl (C14:0), dipalmitoyl (C16:0), and distearoyl (C18:0) PC (Li *et al.,* 2014).

Liposomes can be made up of natural or synthetic phospholipids. Natural phospholipids are found in animal and plant sources such as egg yolk, bovine brain, soybean, and corn. Egg yolk and soybean are the most important sources (Li *et al.,* 2014). Although their price is lower than that of synthetic phospholipids, getting a purified single component of naturally occurring phospholipids is not an easy task. Researches have preferred chemical synthesis, which helps to obtain a single phospholipid component with a defined structure and configuration (Eibl 1980). Synthesis of phospholipids involves the formation of ester or ether bonds to link nonpolar moieties to the backbone followed by the attachment of the polar head group. DMPC is one example of synthetic phospholipids, and it is a di-saturated phospholipid made up of two myristic acids (C14:0), as shown in Figure 3. It is used in research as a model to study liposomes, lipid bilayers, and biological membranes (Van Hoogevest and Wendel 2014).

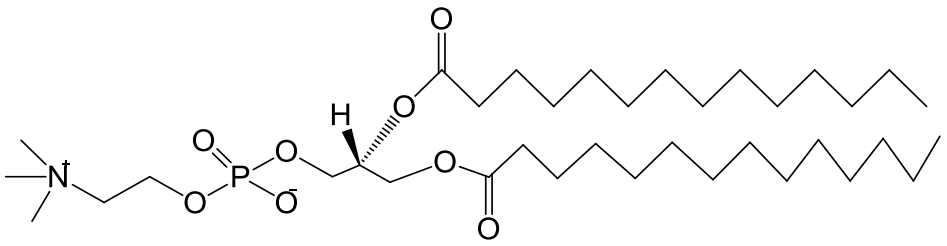


Figure 3: Chemical structure of 1,2-dimyristoyl-sn-glycero-3-phosphocholine.

2. Classification of Liposomes

Liposomes are classified into different groups based on their size and lamellarity, surface charge, and circulation *in vivo*. Liposomes are formed as small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), or large multilamellar vesicles (MLVs). SUVs

are usually 20-50 nm in size and can be generated form MLVs by sonication or by solvent injection methods. LUVs are 100-400 nm in size allowing them to encapsulate large volumes of solution in their cavity. MLVs range from 500 nm to 10 μm and are spontaneously formed when an excess volume of aqueous solution is added to dry lipids (Banerjee 2001).

Liposomes can be positively charged, negatively charged, or neutral depending on the nature of lipid used. Cationic liposomes are preferably used in biomedical applications due to their interaction with the negatively charged cell membrane and nucleic acids (Shim *et al.,* 2013).

Based on their circulation time *in vivo*, liposomes are classified as classical or sterically stabilized. Classical (or conventional) liposomes consist of neutral phospholipids, such as PC, in addition to cholesterol to increase the stability of liposomes in plasma. These liposomes are recognized by phagocytic cells in the body and are rapidly removed from the circulation. Sterically stabilized liposomes are classical liposomes combined with specific molecules, such as polyethylene glycol, to enhance their stability and reduce their reactivity towards plasma membrane proteins and receptors (Banerjee 2001).

3. Methods for Liposome Preparation

A number of methods have emerged throughout the years for the preparation of liposomes. They are categorized into mechanical dispersion methods, solvent dispersion methods, and detergent removal methods. All these methods involve the same basic steps, which consist of drying the lipids from organic solvents, dispersing the lipid in an aqueous media, and purifying the resultant liposomes (Akbarzadeh *et al.,* 2013).

Lipid film hydration is an example of a mechanical dispersion method. The lipids are dissolved in organic solvents, usually a mixture of chloroform and methanol, followed by solvent removal by rotary evaporation. After getting a lipid film, an aqueous medium, such as distilled water or buffer, is added to the dry lipids. The temperature of the hydrating medium should be above the phase transition temperature of the lipid. The resulting liposomes are MLVs, which can be downsized to SUVs by sonication or extrusion (Dua *et al.,* 2012; El Khoury and Patra 2013).

An example of a solvent dispersion method is ethanol injection. A lipid solution of ethanol is rapidly injected to an excess of buffer through a fine needle forming SUVs immediately. Although it is a straightforward method and forms SUVs without the need for sonication, it has its disadvantages including the formation of a heterogeneous solution with size ranging from 30 to 110 nm, difficulty of removing all ethanol, and very diluted liposomes (Batzri and Korn 1973).

Detergents at their critical micelles concentrations are sometimes used to solubilize lipids in solution. As the detergent is removed by a detergent removal method, the micelles become richer in phospholipid and combine to form LUVs. Dialysis is one way to remove the detergent. For this, the lipid-detergent mixed micelles solution is placed in a dialysis bag in a detergent-free aqueous medium allowing the detergent to exist the porous dialysis bag. Although the formed LUVs are of the same size, there is always a trace of detergent within the liposome (Ollivon *et al.,* 2000).

4. Liposomes as a Drug Delivery System

Gregoriadis et al., (1971) were the first to use liposomes to deliver bioactive substances (Gregoriadis *et al.,* 1971). Liposomes serve as delivery systems for anticancer drugs, antifungal drugs, vaccines, and other therapeutic agents (Li *et al.,* 2014). They can also deliver dyes to textiles, supplements to food, and pesticides to plants. Liposomes can be used for the injection of genetic materials into a host cell, a process known as lipofection. In addition to their role in delivery systems, liposomes can serve as models for artificial cells (Dua *et al.,* 2012). Liposomes have the ability to entrap hydrophilic molecules in their aqueous compartment and lipophilic molecules into their membrane. Liposomes can also help reduce drug toxicity and improve its stability and bioavailability. In addition, liposomes possess tissue-targeting properties and maintain the drug intact while delivering it to the site of action (Li *et al.,* 2014).

Liposome-cell interactions can occur through a number of different processes as illustrated in Figure 4. Liposomes can adsorb onto the cell membrane surface. They can fuse with the cell membrane and release their content into the cytoplasm. Liposomes are capable of undergoing an exchange of the lipid components with the cell membrane. They can also be subjected to endocytosis by the cell. Liposomes are delivered into the lysosome by an endosome, where the liposomal membrane is degraded releasing their content (Torchilin 2005).

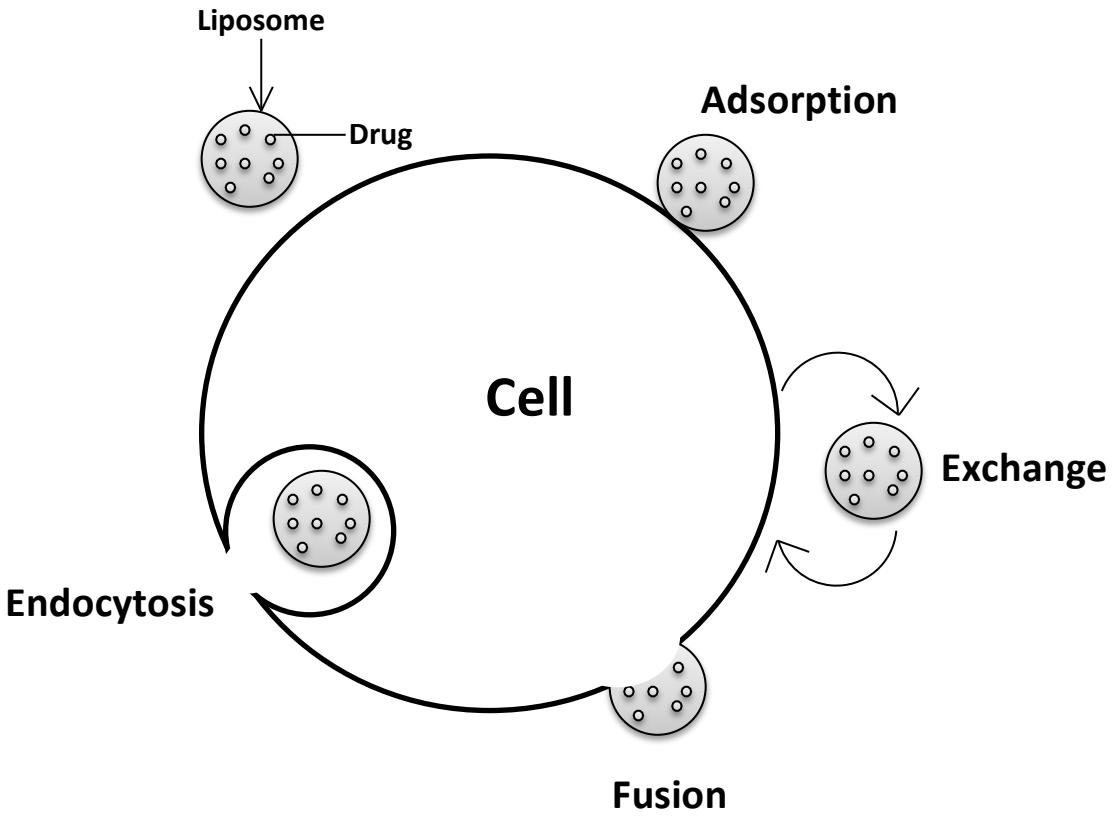


Figure 4: Schematic representation of liposome-cell interactions. Liposomes release their content by interacting with the cell membrane through adsorption, exchange of lipid component materials, fusion, or endocytosis.

CHAPTER III

MATERIALS AND METHODS

A. Materials

DMPC was obtained from Avanti Polar Lipids and used as received. Curcumin and silver nitrate were purchased from Sigma-Aldrich. Chloroform and methanol, from SIAL, were used to prepare liposomes.

For cellular assays, curcumin with 95.2% purity was purchased from Calbiochem and was dissolved in DMSO from Fluka Analytical. HeLa cells were graciously provided by Dr. George Nemer's lab at the American University of Beirut. Dulbecco's Modified Eagle's medium (DMEM), penicillin/streptomycin, and Sodium/Pyruvate were obtained from Lonza. Fetal bovine serum (FBS) was obtained from Sigma-Aldrich.

MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Arcos Organics. Isobutanol from Sigma-Aldrich, sodium dodecyl sulfate (SDS) from Sigma-Aldrich, and HCl were used to prepare MTT stop solution.

B. Methods

1. Preparation of Curcumin-Conjugated AgNPs

Silver nanoparticles were synthesized by dissolving curcumin in double distilled water at 90°C. The stirring was maintained at 90°C and silver nitrate was then added. The solution was kept stirring for around an hour until its color changed from yellow to olive green. Finally, the solution was centrifuged up to 20,000 rpm to sediment the AgNPs. The

AgNPs pellet was then dissolved in double distilled water and stored at 4°C. The size range of the prepared AgNPs was indicated using scanning electron microscopy (SEM).

2. Preparation of DMPC Liposomes

The method used to prepare DMPC liposomes was the lipid film hydration method (Dua et al., 2012; El Khoury and Patra 2013). DMPC phospholipids were dissolved in a mixture of chloroform/methanol (1:1 volume ratio). Using a rotary evaporator, the solvent mixture was evaporated at 60°C, forming a lipid film. Adding 3 mL double distilled water formed liposomes at a concentration of 5 mM. The mixture was then vigorously vortexed and heated at 35°C, which is 10°C above the phase transition temperature of DMPC, until the lipid film was hydrated and formed a homogeneous solution. The prepared liposomes were MLVs. To obtain SUVs, the solution was sonicated for few minutes.

3. Sample Preparation

Samples for each of the mentioned studies were prepared in the absence of AgNPs and in their presence with different concentrations (5, 15, and 20 pM). All fluorescence measurements were done at an excitation wavelength of 425 nm and emission wavelength range of 440-650 nm. To increase its solubility, the stock solution of curcumin was prepared using methanol.

To study the effect of AgNPs on curcumin, samples were prepared with different concentrations of AgNPs, and the concentrations of curcumin and DMPC were fixed at 9 μM and 100 μM, respectively. The same experiment was done by varying the concentration of DMPC.

For partition coefficient studies, samples were prepared with different DMPC concentrations, 9 μM curcumin, and AgNPs.

For quenching studies, samples were prepared with CPB of different concentrations, 15 μM curcumin, 100 μM DMPC, and AgNPs.

For phase transition studies, a sample of 3 μM curcumin and 100 μM DMPC was prepared. Phase transition was studied in the absence and of AgNPs. Fluoresnce was measured at different temperatures.

4. Spectroscopic Measurements

JASCO V-750 UV-VIS-NIR spectrophotometer was used to record absorption spectra at room temperature. For characterization of the silver nanoparticle size, SEM analysis was done using Tescan, Vega 3 LMU with Oxford EDX detector (Inca XmaW20). As for the steady-state fluorescence (emission and excitation) measurements, they were recorded with increment 1 nm and slit 5 nm using Jobin-Yvon-Horiba Flurolog III fluorometer and the FluorEssence program. For the excitation source, a 100 W Xenon lamp was used, and the detector used was R-298 operating at a voltage of 950 V. For temperature regulation, a thermostat was connected to the sample holder, and a thermometer was used to measure the final temperature of the sample.

5. Culture of HeLa cells

HeLa cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% Sodium/Pyruvate. Cells were grown in a 10 mm petri dish

and kept at 37°C in an incubator with a humidified atmosphere containing 95% O_2 and 5% CO_2 until they reached 80-90% confluency.

6. Anti-proliferation Study by MTT Assay

MTT assay was used to measure the cell metabolic activity after treatment with curcumin, AgNPs, or curcumin in combination with AgNPs. HeLa cells were seeded at a density of 3,500 cells per well in 96-well plates. At 50% confluence, cells were subjected to different treatments. Curcumin concentration ranged from 5 μM to 30 μM, AgNPs concentrations were 5, 20, 50, and 100 pM, and Cur-AgNPs were prepared with different concentrations of AgNPs (5, 20, 50, and 100 pM) and 23 μM curcumin. The final concentration of DMSO in culture medium did not exceed 0.1%. Cells were then cultured for 24 and 48 hours. At each time point, 1 mg/mL of MTT dye was added to the cells. After a 3-hour incubation at 37°C, MTT stop solution (10% SDS, 10% isobutanol in 0.01M HCl) was added to solubilize the formazan crystals. The ELISA microplate reader (Thermo/LabSystems 352 Multiskan MS) was used to read the plates at a wavelength of 595 nm.

7. Statistical Analysis

Data are presented as mean values of three different experiments and expressed as mean ± SD. The data were tested for statistical significance by the student's t-test. Significance levels were donated as *($p<0.05$), **($p<0.01$), and ***($p<0.005$).

CHAPTER IV

RESULTS

A. Synthesis of Curcumin-Conjugated AgNPs

Formation of AgNPs was confirmed by UV-visible spectroscopy. LSPR peak of AgNPs is clearly visible at ~420 nm as shown in Figure 5. SEM images show our synthesized AgNPs to have spherical shape and indicate their size to range from ~20 nm to ~40 nm (Figure 6).

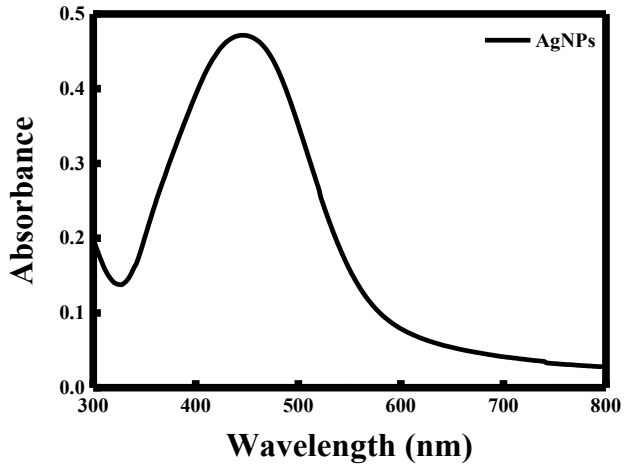


Figure 5: UV-spectrum of curcumin-conjugated AgNPs. LSPR peak of AgNPs is shown at ~420nm.

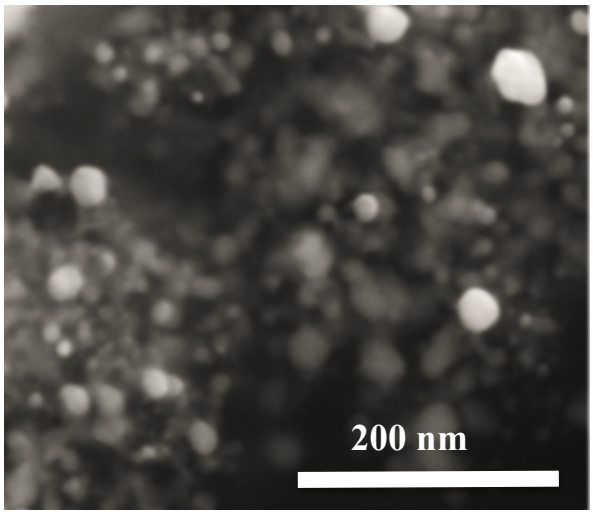


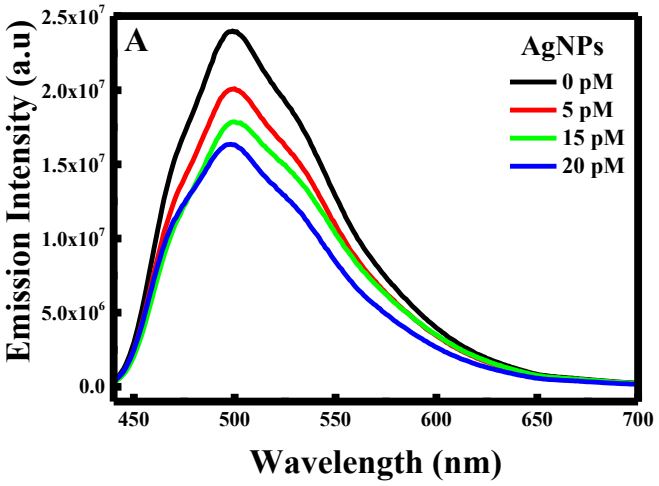
Figure 6: SEM of curcumin-conjugated AgNPs. Formed AgNPs are spherical in shape.

B. Quenching Effect of AgNPs on Curcumin

AgNPs was shown to have a fluorescence quenching effect on curcumin. As the concentration of AgNPs increases, fluorescence intensity of curcumin decreases (Figure 7).

C. DMPC Liposomes Decrease Quenching of Curcumin by AgNPs

Adding DMPC liposomes to the curcumin-AgNPs solution restores the fluorescence intensity of curcumin. As the concentration of DMPC liposomes increases, the fluorescence intensity of curcumin increases as shown in Figure 8.



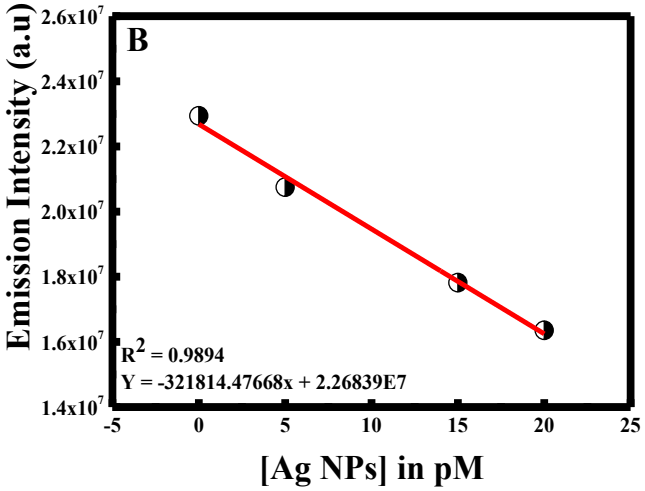


Figure 7: AgNPs have a quenching effect on curcumin. **(A)** Fluorescence emission spectra of curcumin in the presence of different AgNPs concentrations. **(B)** Linear fit showing the decrease in emission maxima of curcumin as the concentration of AgNPs increases.

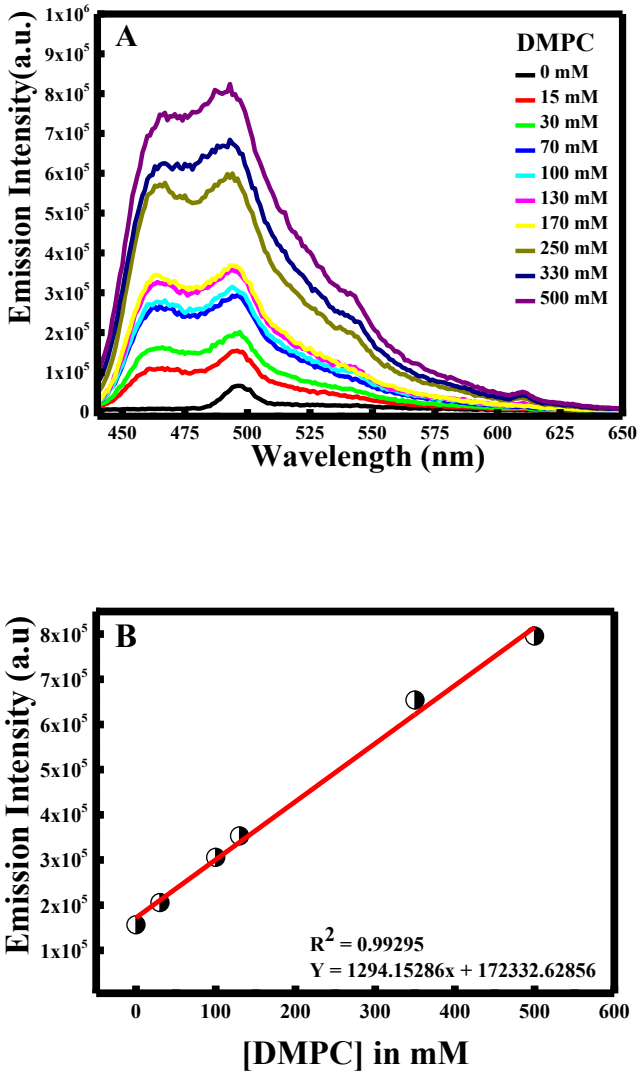


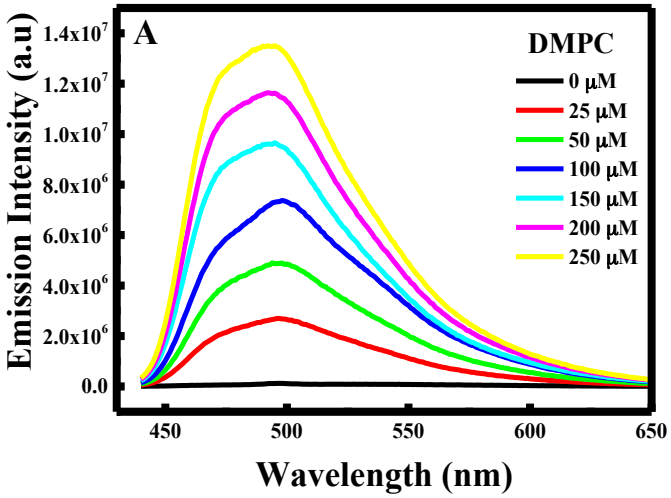
Figure 8: DMPC liposomes restore fluorescence intensity of curcumin in the presence of AgNPs. **(A)** Fluorescence emission spectra of curcumin in the presence of different DMPC liposome concentrations. **(B)** Linear fit showing the increase in emission maxima of curcumin as the concentration of DMPC liposomes increases.

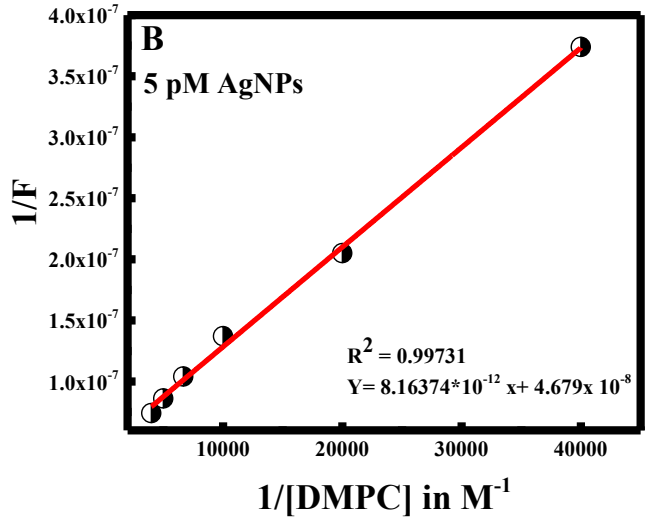
D. Effect of AgNPs on the Partition of Curcumin into DMPC Liposomes

Partition coefficient (Kp) was used as a mean to study the effect of AgNPs on DMPC permeability using curcumin as a probe molecule. As the concentration of DMPC increases, the fluorescence intensity of curcumin increases and the emission maximum shifts to a shorter wavelength (Figure 9A). Addition of AgNPs changes the partition coefficient of DMPC (Figure 9C). Increasing the concentration of AgNPs decreases the Kp value as shown in Table 1.

Curcumin in DMPC	**Partition coefficient (Kp)**
no AgNPs	1.15×10^{6} (El Khoury and Patra 2013)
5 pM AgNPs	3.19×10^{5}
15 pM AgNPs	4.12×10^{4}
20 pM AgNPs	3.81×10^{4}

Table 1: Partition coefficients of curcumin into DMPC liposomes in the absence and presence of AgNPs


A
DMPC
0 µM
25 µM
50 µM
100 µM
150 µM
200 µM
250 µM
Emission Intensity (a.u)
1.4x10^7
1.2x10^7
1.0x10^7
8.0x10^6
6.0x10^6
4.0x10^6
2.0x10^6
0.0
450
500
550
600
650
Wavelength (nm)


B
5 pM AgNPs
R^2 = 0.99731
Y= 8.16374*10^-12 x+ 4.679x 10^-8
1/F
4.0x10^-7
3.5x10^-7
3.0x10^-7
2.5x10^-7
2.0x10^-7
1.5x10^-7
1.0x10^-7
10000
20000
30000
40000
1/[DMPC] in M^-1

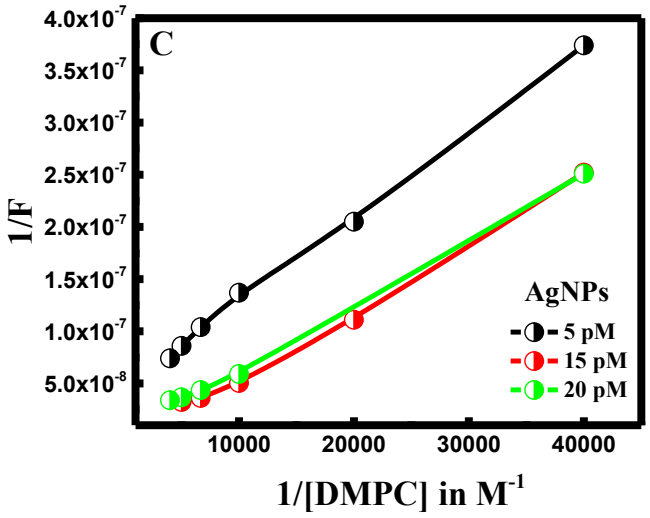


Figure 9: AgNPs affect the partition of curcumin into DMPC liposomes. **(A)** Fluorescence emission spectra of DMPC – curcumin system in the presence of 5 pM AgNPs and different DMPC concentrations. **(B)** Double-reciprocal plot 1/F versus 1/[DMPC] in the presence of 5 pM AgNPs. **(C)** Double-reciprocal plot 1/F versus 1/[DMPC] of DMPC – curcumin system in the presence of different AgNPs concentrations.

E. Effect of AgNPs on the Fluorescence Quenching of Curcumin by CPB in DMPC Liposomes

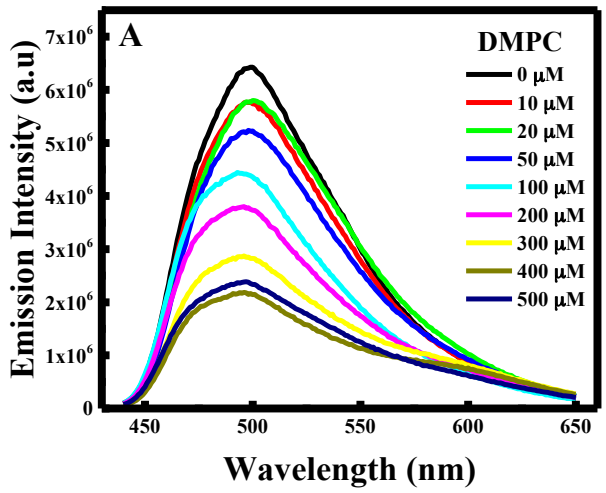
Stern-Volmer constant (KSV) was used as a mean to study the location of curcumin and AgNPs in DMPC liposomes. As shown in Figure 10A, the fluorescence intensity of curcumin decreases as the concentration of CPB increases. Increasing the concentration of AgNPs increases the K_{SV} value as shown in Table 2.

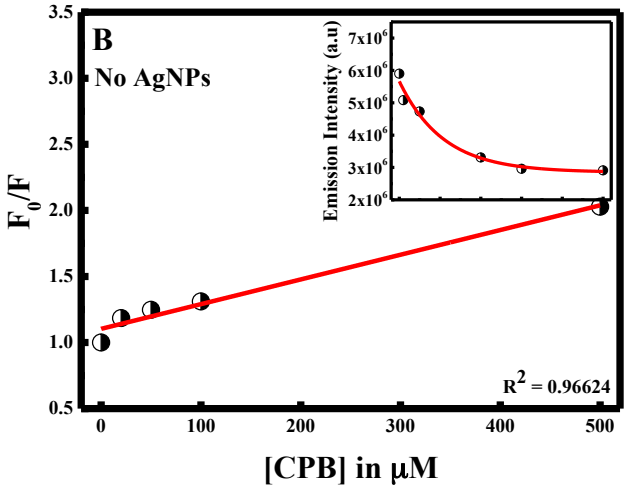
Curcumin in DMPC	Stern-Volmer constant (K_{SV})
no AgNPs	1.187×10^{-3}
5 pM AgNPs	3.97×10^{-3}
15 pM AgNPs	25.31×10^{-3}
20 pM AgNPs	20.42×10^{-3}

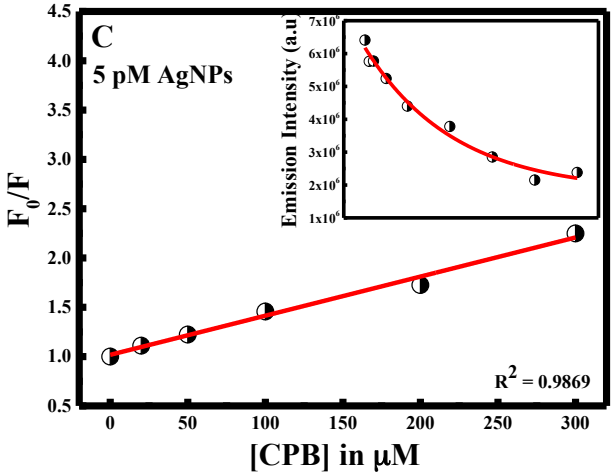
Table 2: Stern-Volmer constants of curcumin quenching by CPB in DMPC liposomes in the absence and presence of AgNPs.

F. Effect of AgNPs on Phase Transition Temperature of DMPC Liposomes

AgNPs alter the pre-transition temperature and phase transition temperature of DMPC, 10°C and 23°C, respectively. It is clear in Figure 11 that in the presence of 5 pM AgNPs, the pre-transition temperature does not change, but the phase transition temperature shifts to 27°C. However, as the concentration of AgNPs increases, pre-transition temperature disappears and the phase transition temperature becomes broader ranging from ~20°C to ~35°C.


A
DMPC
0 µM
10 µM
20 µM
50 µM
100 µM
200 µM
300 µM
400 µM
500 µM
Emission Intensity (a.u)
Wavelength (nm)


B
No AgNPs
F_0/F
[CPB] in µM
R^2 = 0.96624
Emission Intensity (a.u)


C
5 pM AgNPs
F_0/F
[CPB] in µM
R^2 = 0.9869
Emission Intensity (a.u)

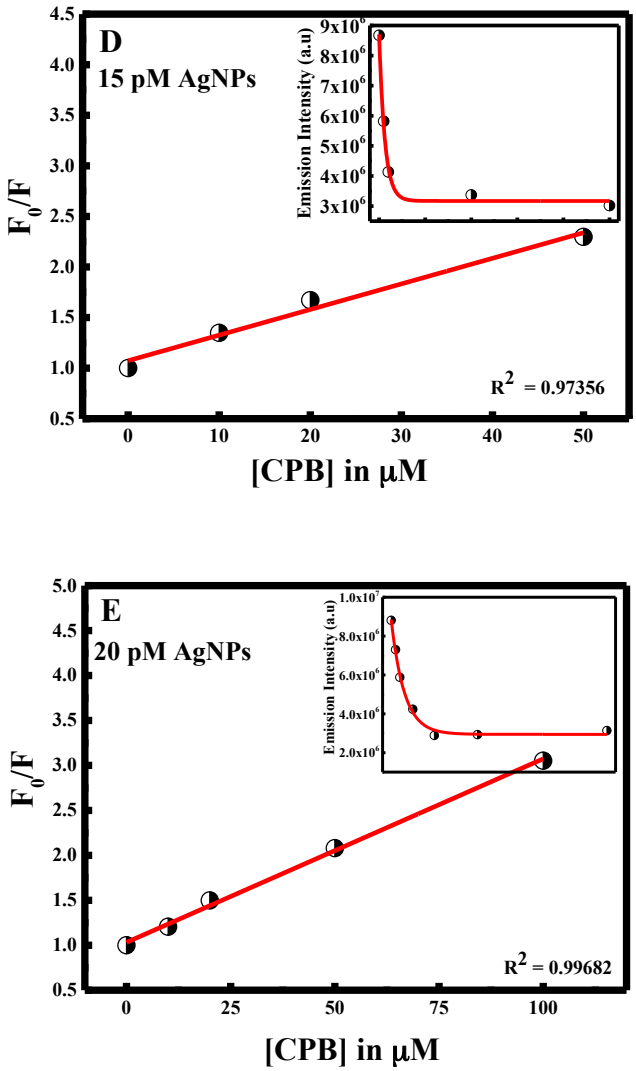


Figure 10: AgNPs affect the quenching of curcumin by CPB in DMPC liposomes. **(A)** Fluorescence emission spectra of DMPC – curcumin system in the presence of 5 pM AgNPs and different CPB concentrations. Linear Stern-Volmer plots are shown in the absence **(B)** and presence of different concentrations of AgNPs: 5 pM **(C)**, 15 pM **(D)**, and 20 pM **(E)**. Insets indicate the decrease in curcumin intensity as CPB concentration increases.

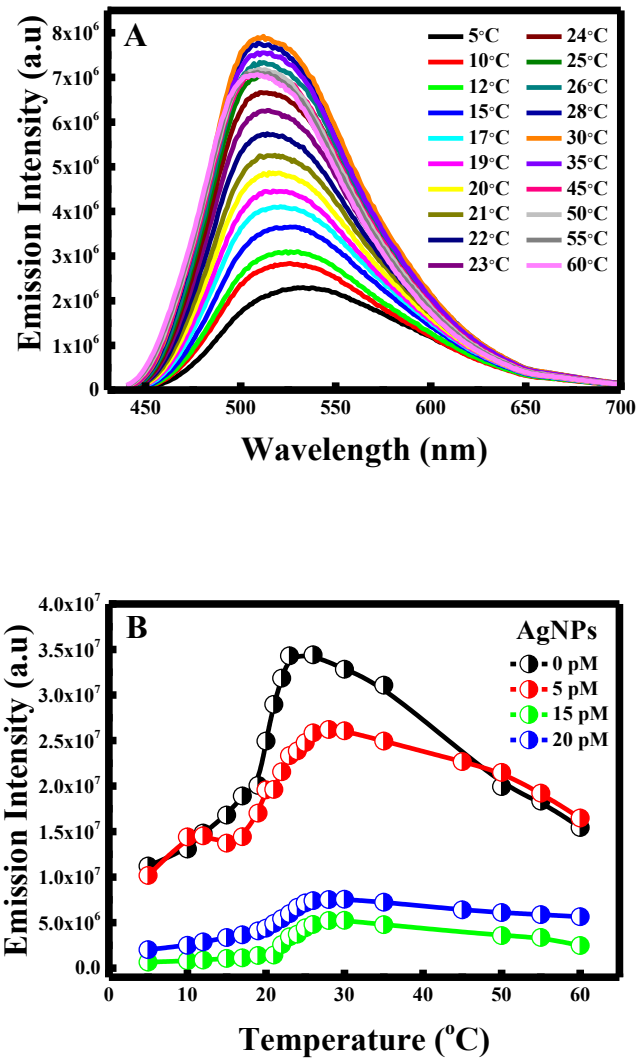


Figure 11: AgNPs broaden the phase transition temperature of DMPC liposomes. **(A)** Fluorescence emission spectra of DMPC – curcumin system at different temperatures. **(B)** Profile of temperature – fluorescence intensity of curcumin at various concentrations of AgNPs in DMPC membranes.

G. Anti-proliferative Effect of Curcumin against HeLa Cells

As indicated in Figure 12, curcumin has time- and concentration-dependent cytotoxic effect on the proliferation of HeLa cells. IC_{50} was calculated and found to be ~23 µM after treatment for 48 hours.

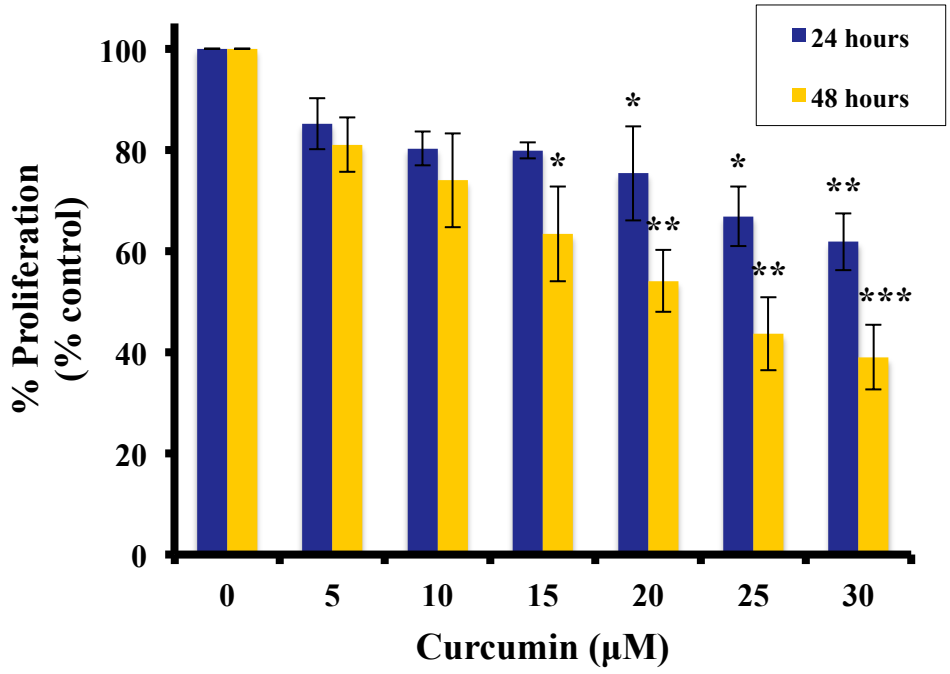


Figure 12: Curcumin has a cytotoxic effect against HeLa cells. Each value is the mean ± SD of 3 independent replicates. Significance levels are donated as *($p<0.05$), **($p<0.01$), and ***($p<0.005$).

H. Anti-proliferative Effect of AgNPs against HeLa Cells

AgNPs have a time- and concentration-dependent cytotoxic effect on the proliferation of HeLa cells as shown in Figure 13.

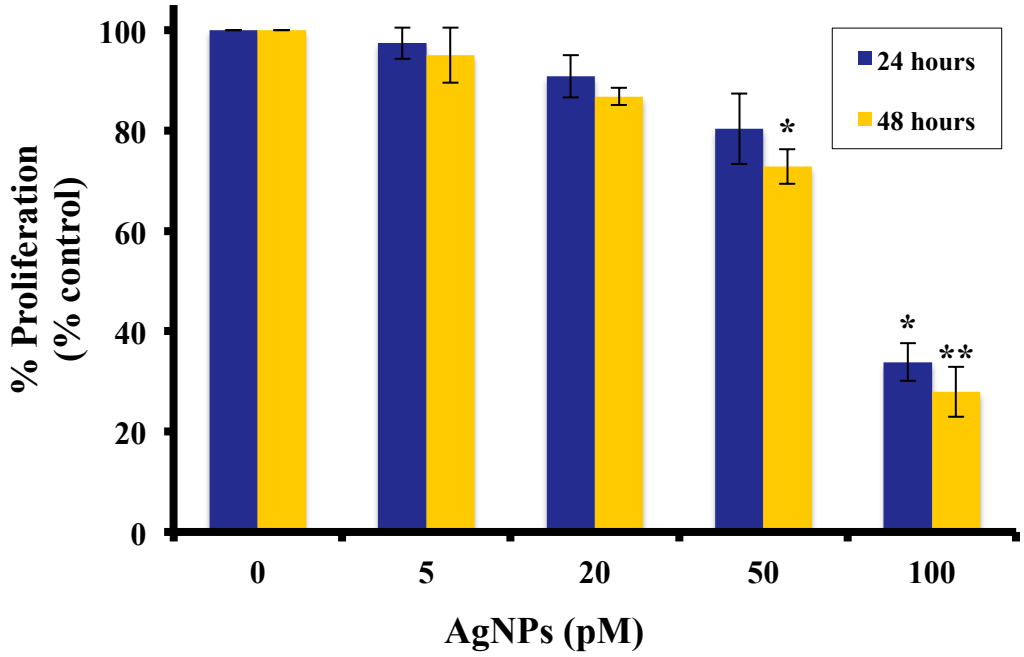


Figure 13: AgNPs have a cytotoxic effect against HeLa cells. Each value is the mean ± SD of 3 independent replicates. Significance levels are donated as *($p<0.05$), **($p<0.01$), and ***($p<0.005$).

I. Anti-proliferative Effect of Cur-AgNPs against HeLa Cells

The combination of curcumin and AgNPs has a time- and concentration-dependent anti-proliferative effect on HeLa cells as indicated in Figure 14. Their effect is found to be higher than that of curcumin or AgNPs alone.

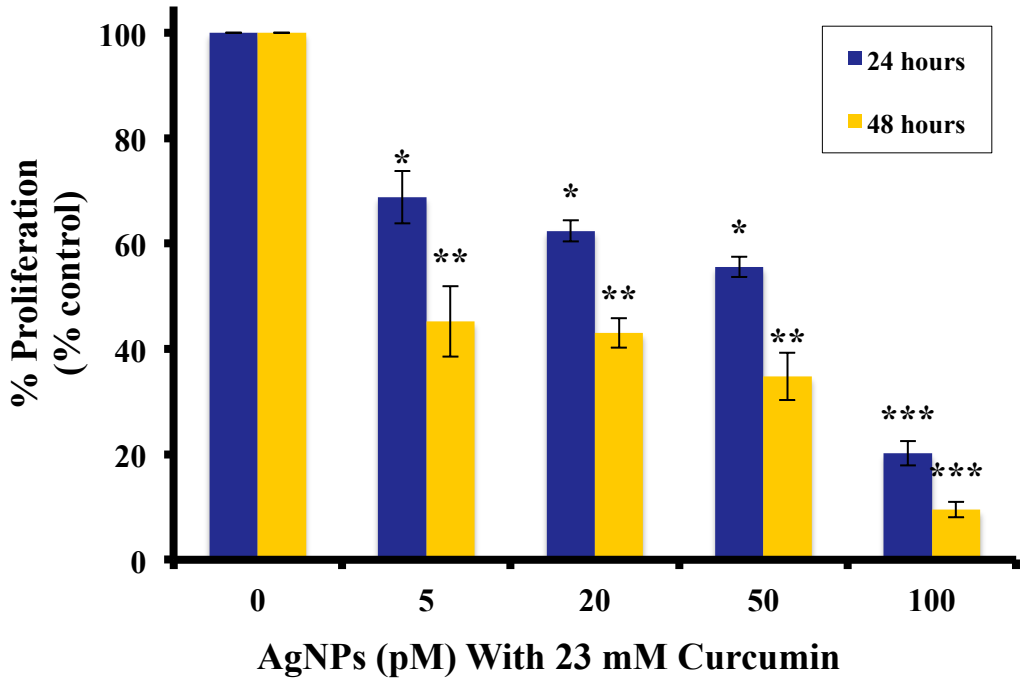


Figure 14: Cur-AgNPs have a cytotoxic effect against HeLa cells. Each value is the mean ± SD of 3 independent replicates. Significance levels are donated as *($p<0.05$), **($p<0.01$), and ***($p<0.005$).

CHAPTER V

DISCUSSION

The solubility and stability of curcumin was shown to improve when encapsulated within membranes. In addition, curcumin has been established as a liposome probe to investigate membrane properties such as fluidity and phase transition temperature (El Khoury and Patra 2016). Therefore, the aim of the present work is to investigate the modulation of phospholipid membranes properties, such as fluidity, permeability, and phase transition temperature, by AgNPs using curcumin as a probe molecule. Furthermore, curcumin is known to be cytotoxic to cervical cancer cells (HeLa). Therefore, cytotoxic effect of curcumin, AgNPs, and curcumin combined with AgNPs on the proliferation of HeLa cells was examined.

A. Formation of Curcumin-Conjugated AgNPs

Recently, several studies have used curcumin as a reducing agent for the green synthesis of AgNPs (Verma *et al.,* 2016; Garg and Garg 2018). As indicated in figure 5, the LSPR peak of our synthesized AgNPs was found to be ~420 nm, which is in accordance with previous studies showing the absorption of spherical AgNPs in the UV-visible spectra at around 400-420 nm (Shameli *et al.,* 2012). Since it is well established that curcumin absorbs in the UV-visible region at ~266 nm and ~426 nm in aqueous media (Khopde *et al.,* 2000; Adhikary *et al.,* 2009), our UV-visible absorption data indicates the association of AgNPs with curcumin. To ensure that the peak is for the synthesized AgNPs and not

curucmin, the measurement of UV-visible absorption was done after separation of curcumin-conjugated AgNPs from unreacted curcumin by centrifugation. SEM images (Figure 6) revealed the spherical shape of our synthesized curcumin-conjugated AgNPs. In addition, the images gave insight about the size of AgNPs ranging from ~20 nm to ~40 nm confirming their nanostructure.

B. DMPC Liposomes Diminish Quenching Effect of AgNPs on Curcumin

Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. Our results showed that as the concentration of AgNPs increased, the amount of free curcumin decreased as indicated by the trend of fluorescence emission spectra in Figure 7. This suggests that AgNPs are interacting with curcumin forming a complex. Adding DMPC liposomes to the solution helped recover the fluorescence intensity of curcumin as shown in Figure 8. The recovery of curcumin's fluorescence was found to be increasing proportionally to the concentration of DMPC. More recovery took place as the concentration of DMPC increased. This suggests that curcumin on the surface of AgNPs is stabilized by DMPC liposomes. In addition, curcumin is known to have a fluorescence emission maximum at ~550 nm in polar media such as water (Patra and Barakat 2011). The blue shift in the fluorescence spectra of curcumin to ~500 nm in the presence of DMPC further indicates that curcumin is interacting with the hydrophobic region of DMPC with high affinity. These results are similar to previous findings in our lab (El Khoury and Patra 2016). The interaction of AgNPs with curcumin and curcumin's high affinity toward DMPC liposomes give a mean to study the effect of AgNPs on phospholipid membranes properties using curcumin as a probe.

C. AgNPs Decrease the Partition of Curcumin into DMPC Liposomes

To investigate membrane permeability, curcumin was used as a probe molecule since it is well known that the fluorescence of curcumin enhances when it binds to membranes compared to in water. The observed increase in fluorescence intensity of curcumin as the concentration of DMPC liposomes is increasing confirms incorporation of curcumin into the membrane. In addition, the blue shift (~50 nm) of emission maximum gives further insight that curcumin is buried into the lipid bilayer of DMPC liposomes. These results are in accordance to previous findings in our lab (El Khoury and Patra 2013). Therefore, to understand the effect of AgNPs on membrane permeability, partition coefficient of curcumin into DMPC liposomes was estimated in the presence of AgNPs.

Partition coefficient (Kp) is used to estimate the lipophilicity of a compound by giving information about the association of the compound with the lipid. The difference between fluorescence intensity of curcumin in aqueous medium and after its incorporation into liposomes could be used to evaluate Kp. The partition coefficient of curcumin into DMPC is expressed as (Huang and Haugland 1991)

$$Kp = (Curb / DMPC) / (Curf / W)$$

where Curb, DMPC, Curf, and W refer to moles of the membrane-associated curcumin, phospholipid membrane, free curcumin in aqueous phase, and water, respectively.

In the present work, we estimated Kp by using the following equation

$$1/F = [55.6/(KpF_0)](1/DMPC) + 1/F_0$$

where F_0 is the maximum fluorescence resulting from total incorporation of curcumin into DMPC membrane and 55.6 is the molar concentration of pure water since concentration of DMPC used is less than 0.2% of the total volume. This equation indicates that a double-

reciprocal plot of the fluorescence intensity and the lipid concentration should give a linear curve. The Kp value could be estimated from the line's slope and intercept. A 1/F vs. 1/[DMPC] plot was used to estimate partition coefficient of curcumin into DMPC liposomes as shown in Figure 9 B&C.

The partition coefficient of curcumin into DMPC liposomes in its liquid crystalline phase was previously estimated to be 1.15E+06 (El Khoury and Patra 2013). As the concentration of AgNPs increase, the partition coefficient of curcumin decreases as shown in Table 1. This establishes that the studied concentrations of AgNPs were able to influence the partition of curcumin deep into the hydrophobic tails of DMPC liposomes therefore affecting membrane's permeability. As depicted in Figure 15, AgNPs are hydrophilic molecules that are found at the surface of the liposomes, they could possibly block the entrance of curcumin into the membrane. Another possibility is that AgNPs might increase spacing between hydrocarbon chains causing curcumin molecules to either move toward the surface in close proximity to the head groups or come out entirely of the lipid bilayer.

D. AgNPs Increase the Quenching of Curcumin by CPB in DMPC Liposomes

To further establish location of curcumin and AgNPs in DMPC liposomes system, fluorescence quenching study of curcumin as probe was applied by using a hydrophobic quencher molecule such as CPB. Membrane permeability is associated with the passive transport across cell membranes and the capability of a compound to partition and intercalate into a membrane. Changes in the membrane permeability are reflected in changes in the rate constant of the quenching reaction or ease of entry of the quencher to the probe, which is usually incorporated within the membrane hydrophobic core.

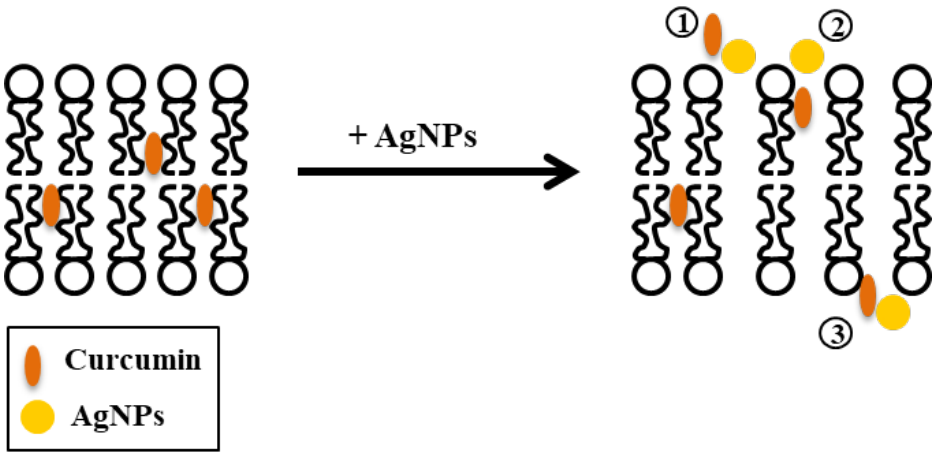


Figure 15: Schematic representation showing the effect of AgNPs on the partition of curcumin into DMPC liposomes: AgNPs could block curcumin's entrance into the membrane **(1)** or increase the space between hydrocarbon chains causing curcumin to move upward in the bilayer **(2)** or exit the bilayer **(3)**.

CPB quenches the fluorescence of curcumin by electron transfer process, where electron in the excited state is transferred from the aromatic ring of curcumin to electron deficient N-atom of CPB (Chebl et al., 2016). In liposomes, CPB intercalates within the hydrophobic part of the membrane with its charged moiety exposed at the surface. Therefore, liposomes encourage contact between CPB and hydrophobic probe like curcumin.

The fluorescence intensity of curcumin was quenched by CPB in the absence and presence of AgNPs in DMPC liposomes. Under steady state conditions, the relationship derived by Stern and Volmer describes quenching as

$F_0/F = 1 + K_{SV}\,[CPB]$

where F_0 and F are the fluorescence intensities in the absence and presence of different CPB concentration ([CPB]), respectively, and Ksv is the Stern-Volmer quenching constant. As shown in Figure 10B-E, the Stern-Volmer plot for fluorescence quenching of curcumin by CPB in DMPC liposomes was found to be linear in low concentration range and at higher concentration of CPB it is saturated as expected due to quenching sphere action as depicted in insets of Figure 10B-E. K_{SV} remarkably increased in the presence of 5 and 15 pM AgNPs in DMPC liposomes; however, with further increase in AgNPs concentration the K_{SV} slightly decreased as summarized in Table 2. This is logical as the presence of AgNPs helps in opening the membrane interface and allows curcumin to come out from deep within the membrane. Thus, curcumin is now located close to the stern layer of liposomes reducing the distance between curcumin and N^+-atom of CPB as shown in Figure 16. This increases K_{SV} since larger distance between curcumin and N^+-atom of CPB reduces the quenching rate. However, additional increase in AgNPs concentration could further expose curcumin to the aqueous phase by decreasing partition of curcumin into the hydrophobic bilayer.

E. AgNPs Broaden the Phase Transition Temperature of DMPC Liposomes

Phase transition is an important aspect of phospholipid membranes since it affects various important functions of the cell. Membranes can exist in a rigid solid gel phase or a more fluid liquid crystalline phase. Phase transition temperature (Tm) is the temperature at which membranes shift from one phase to another. This phenomenon regulates cell membranes fluidity and permeability. Factors that affect Tm include the nature of the polar head group,

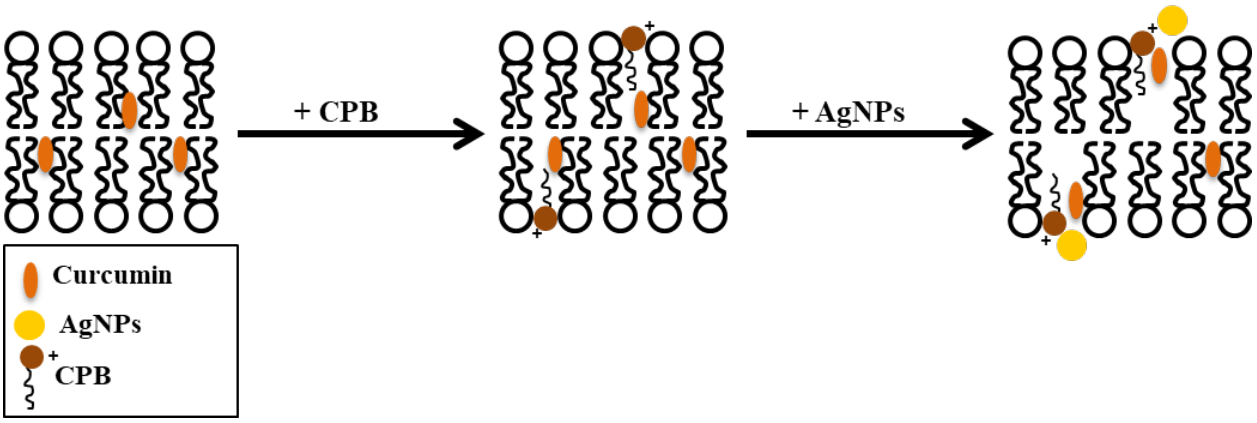


Figure 16: Schematic representation showing the effect of AgNPs on the quenching of curcumin by CPB in DMPC liposomes. The presence of AgNPs opens up the membrane interface causing curcumin to come out close to the stern layer of liposomes; thus, increasing the interaction between curcumin and N^+-atom of CPB.

length of the hydrocarbon chains, and degree of saturation of the hydrocarbon chains. Long and highly saturated hydrocarbon chains tend to have a higher Tm. In addition, the purity of phospholipids affects the transition temperature. Naturally occurring phospholipids tend to have a broader range of Tm as compared to synthetic phospholipids, which usually have a definite Tm (Li *et al.,* 2014).

Fluorescence is a good method to study phase transition since it is highly sensitive to low concentrations hence requires small samples; therefore, our lab has recently established using curcumin as a fluorescence probe to detect phase transition temperature of membranes (Patra *et al.,* 2012). In general, phase transition consists of two phases: pre-transition and the main phase transition. For DMPC liposomes, it is known that pre-transition occurs at ~10°C when DMPC membranes undergo a change from solid gel phase

to rippled gel phase, and the main phase transition occurs ~23°C where the rigid gel phase of DMPC membranes transforms into the more fluid liquid crystalline phase (Eklund *et al.,* 1984) as illustrated in Figure 17.

As shown in Figure 11A, as temperature increases, the fluorescence intensity of curcumin in DMPC increased to a maximum at Tm reconfirming that curcumin can be successfully used to the study the phase transition of membranes. After Tm, the intensity decreased as the system became homogeneous at the liquid phase. Such behavior of fluorescence intensity of curcumin in DMPC liposomes with temperature is related to the changes in the permeability and fluidity of the DMPC liposomes. As the system is shifting from the dense solid gel phase to the less compacted liquid crystalline phase, membrane permeability increases. Therefore, more curcumin can penetrate the DMPC liposomes resulting in an enhancement in the fluorescence intensity of curcumin. Besides the permeability of the membrane, another factor should be considered which is the microviscosity or the fluidity of the environment. A decrease in the microsviscosity or an increase in the fluidity of the membrane accounts for the decrease in the emission intensity after attaining the phase transition temperature. As a result, the fluorescence intensity of curcumin was dominated by the permeability factor prior to Tm and by the mircoviscosity or fluidity factor after it.

AgNPs were found to alter both the pre-transition temperature and the phase transition temperature of DMPC liposomes. As depicted in Figure 11B, as the concentration of AgNPs increases, the pre-transition temperature is not detected anymore and the phase transition temperature becomes broader (20 to 35°C). This finding suggests that the presence of AgNPs at the interface of the membrane's head groups results in the

fusion of the solid gel and the liquid crystalline phases shown in Figure 16. AgNPs provided DMPC liposomes with properties of biological membranes. Since biological membranes are a complex mixture of different lipids each having its unique Tm, at a given temperature, some lipids will be in the solid gel phase and others in liquid crystalline phase. The coexistence of the two phases results in a phenomenon known as phase separation, where the two phases exist in spatially separated populations. This could result in aggregation and/or activation of certain proteins in one of the phases. On the other hand, increase in the spacing between hydrocarbon chains may lead to the leakage of cytoplasmic contents out of the cell (Heberle and Feigenson 2011). Therefore, this could be another mean by which AgNPs mediate their cytotoxicity. In addition, the use of AgNPs-conjugated liposomes as drug delivery system could help increase the drug release rate from the aqueous compartment of the liposomes.

F. Curcumin has an Anti-proliferative Effect against HeLa Cells

MTT proliferation assay was used to measure anti-proliferative effect of curcumin on HeLa cells. As shown in Figure 12, curcumin has time- and concentration-dependent anti-proliferative effect on HeLa cells. Up to 60% inhibition in proliferation of HeLa cells was observed after 48-hour treatment with 30 μM. Low concentrations of curcumin, 5 and 10 μM, were shown to inhibit cell proliferation by 19% and 26%, respectively. IC_{50} was calculated and found to be 22.7±3.28 μM after treatment with curcumin for 48 hours. Several previous studies established the anti-proliferative effect of curcumin on cervical cancer cells.

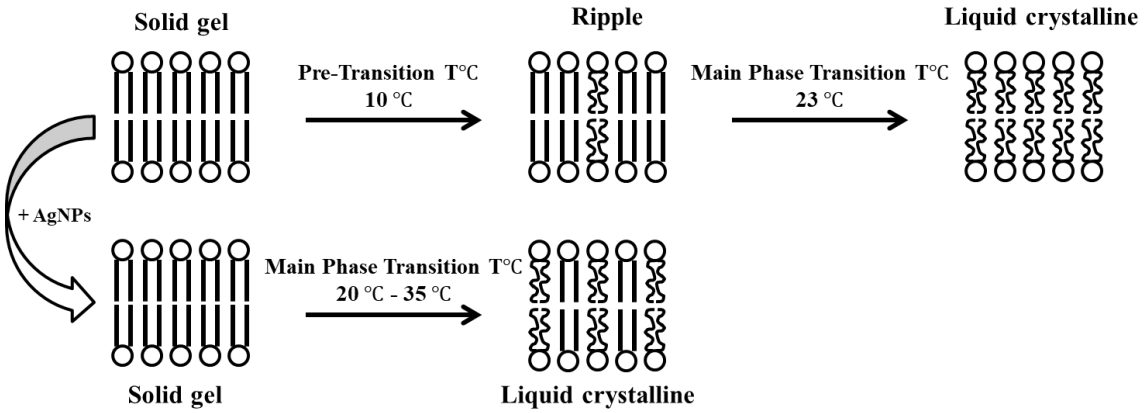


Figure 17: Schematic representation showing the effect of AgNPs on the phase transition temperature of DMPC liposomes. The presence of AgNPs diminishes pre-transition temperature and broadens the main transition temperature causing the fusion of solid gel and liquid crystalline phases.

One study estimated IC_{50} of curcumin to be 17.67±1.10 μM after treatment for 72 hours (Ding *et al.,* 2015). Another recent study calculated IC_{50} to be 25±5.2 μM, which is similar to what we obtained (Thacker and Karunagaran 2015). The same study found that combination of curcumin and emodin, a compound present in the roots and barks of several medicinal plants, synergistically inhibited proliferation of HeLa cells. This suggested studying the cytotoxic effect of combining curcumin and our synthesized AgNPs on the proliferation of HeLa cells.

G. AgNPs Enhance Anti-proliferative Effect of Curcumin against HeLa Cells

AgNPs are known to exhibit anticancer, antibacterial, and antifungal activities. Several studies established the effective anticancer activity of green synthesized AgNPs. In

one study, AgNPs were synthesized using *Moringa oleifera* stem bark extract. A concentration of 250 µg/mL was shown to decrease the viability of HeLa cells by 94% after incubation for 24 hours. These AgNPs were shown to mediate their effect by inducing apoptosis through the generation of ROS (Vasanth *et al.,* 2014). Another study demonstrated the use of marine bacteria, *Escherichia coli*, to synthesize AgNPs. These AgNPs were shown to exhibit cytotoxic effect against HeLa cells at a concentration range of 25-125 µg/mL. A concentration of 125 µg/mL caused 85.36% decrease in the proliferation of HeLa cells after treatment for 24 hours (Viswanathan *et al.,* 2016). A recent study used curcumin for the green synthesis of AgNPs. The synthesized curcumin-AgNPs were found to be cytotoxic against breast and lung cancer cells after treatment of 10, 20, 40, and 80 µg/mL for 24 hours (Garg and Garg 2018).

MTT proliferation assay was used to measure anti-proliferative effect of our curcumin-conjugated AgNPs on HeLa cells. As shown in Figure 13, picomolar concentrations of AgNPs have time- and concentration-dependent anti-proliferative effect on HeLa cells. Up to 70% inhibition in proliferation of HeLa cells was observed after 48-hour treatment with 100 pM. The combination of curcumin (IC_{50}) with AgNPs showed synergistic inhibition against the proliferation of HeLa cells as indicated in Figure 14. Combination of curcumin with 100 pM AgNPs showed ~ 90% inhibition, which is 1.8-fold increase compared to curcumin alone. Using AgNPs with curcumin enhances the anticancer activity of curcumin. Therefore, this combination could be used as a potential anticancer agent against various cancer types.

CONCLUSION

AgNPs are well known for their wide use in biological and environmental applications, but their potential toxicity is still controversial. Therefore, further research is required to understand the effect of these nanoparticles *in vitro* before applying them *in vivo*. Our present work revealed that AgNPs affect some properties of DMPC liposome, a model for phospholipid membranes. AgNPs decreased the partition of curcumin, a molecular probe with high safety profile, into DMPC liposomes. They were also found to broaden the phase transition temperature of DMPC liposomes. The combination of AgNPs and curcumin enhanced the anti-proliferative effect of curcumin against HeLa cells. Delivering AgNPs along with curcumin in DMPC liposomes could be used as a vehicle system for the potential treatment of cancer.

REFERENCES

Abou El-Nour, K., Eftaiha, A., Al-Warthan, A., & Ammar, R. (2010). "Synthesis and Applications of Silver Nanoparticles." *Arabian Journal of Chemistry*, 3(3): 135–140. doi: 10.1016/j.arabjc.2010.04.008

Adhikary, R., Mukherjee, P., Kee, T., & Petrich, J. (2009). "Excited-State Intramolecular Hydrogen Atom Transfer and Solvation Dynamics of the Medicinal Pigment Curcumin." *J. Phys. Chem.*, 12: 5255–5261. doi: 10.1021/jp901234z

Aggarwal, B., Shishodia, S., Takada, Y., Banerjee, S., Newman, R., Bueso-Ramos, C., & Price, J. (2005). "Curcumin Suppresses the Paclitaxel-Induced Nuclear Factor-κB Pathway in Breast Cancer Cells and Inhibits Lung Metastasis of Human Breast Cancer in Nude Mice." *Clinical Cancer Research*, 11(20): 7490–7498. doi: 10.1158/1078-0432.CCR-05-1192

Aggarwal, B., Sundaram, C., Malani, N., & Ichikawa, H. (2007). "Curcumin : The Indian Solid Gold." In: Aggarwal B.B., Surh YJ., Shishodia S. (eds) The Molecular Targets and Therapeutic Uses of Curcumin in Health and Disease. ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY, vol 595. Springer, Boston, MA

Akbarzadeh, A., Rezaei-Sadabady, R., Davaran, S., Joo, S., Zarghami, N., Hanifehpour, Y., Samiei, M., Kouhi, M., & Nejati-Koshki, K. (2013). "Liposome: Classification, Preparation, and Applications." *Nanoscale Research Letters*, 8(1): 1–8. doi: 10.1186/1556-276X-8-102

Alao, J. (2007). "The Regulation of Cyclin D1 Degradation: Roles in Cancer Development and the Potential for Therapeutic Invention." *Molecular Cancer*, 6(24): 1–16. doi:10.1186/1476-4598-6-24

Anand, P., Kunnumakkara, A., Newman, R., & Aggarwal, B. (2007). "Bioavailability of Curcumin : Problems and Promises Reviews Bioavailability of Curcumin : Problems and Promises." *Molecular Pharmacology*, 4(6): 807–818. doi: 10.1021/mp700113r

Aqil, F., Munagala, R., Jeyabalan, J., Agrawal, A., & Gupta, R. (2017). "Exosomes for the Enhanced Tissue Bioavailability and Efficacy of Curcumin." *The AAPS Journal*, 19(6): 1691–1702. doi: 10.1208/s12248-017-0154-9

Asanithi, P., Chaiyakun, S., & Limsuwan P. (2012). "Growth of Silver Nanoparticles by DC Magnetron Sputtering." *Journal of Nanomaterials,* 2012: 1–8. doi: 10.1155/2010/963690

Asharani, P., Sethu, S., Lim, H., Balaji, G., Valiyaveettil, S., & Hande, M. (2012). "Differential Regulation of Intracellular Factors Mediating Cell Cycle, DNA Repair and Inflammation Following Exposure to Silver Nanoparticles in Human Cells." *Genome Integrity*, 3(2): 1–14. doi: 10.1186/2041-9414-3-2

Banerjee, R. (2001). "Liposomes: Applications in Medicine." *Journal of Biomaterials Applications*, 16(1): 3–21. doi: 10.1106/RA7U-1V9C-RV7C-8QXL

Bangham, A., & Horne, R. (1964). "Negative Staining of Phospholipids and Their Structural Modification by Surface-Active Agents as Observed in the Electron Microscope." *Journal of Molecular Biology*, 8(5): 660–68. doi: 10.1016/S0022-2836(64)80115-7

Batzri, S., & Korn, E. (1973). "Single Bilayer Liposomes Prepared without Sonication." *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 298(4): 1015–1019. doi: 10.1016/0005-2736(73)90408-2

Bharti, A., Donato, N., Singh, S., & Aggarwal, B. (2003). "Curcumin (diferulolmethane)down-Regulates the Constitutive Activation of Nuclear factor–κB and IκBα Kinase in Human Multiple Myeloma Cells, Leading to Suppression of Proliferation and Induction." *American Society of Hematology*, 101(3): 1053–1062. doi: 10.1182/blood-2002-05-1320

Biswas, S. (2016). "Does the Interdependence between Oxidative Stress and Inflammation Explain the Antioxidant Paradox?" *Oxidative Medicine and Cellular Longevity*, 2016: 17–26. doi: 10.1155/2016/5698931

Chainani-Wu, N. (2003). "Safety and Anti-Inflammatory Activity of Curcumin: A Component of Tumeric (*Curcuma Longa*)." *The Journal of Alternative and Complementary Medicine*, 9(1): 161–168. doi: 10.1089/107555303321223035.

Chebl, M., Abiad, M., Moussa, Z., & Patra, D. (2016). "Two Modes of Associations of Curcumin with Pre- and Nanoaggregated Chitosan Oligosaccharide Lactate: Ionic Strength and Hydrophobic Bile Salt Modulate Partition of Drug and Self-Assembly Process." *Journal of Physical Chemistry C*, 120(20): 11210–11224. doi: 10.1021/acs.jpcc.6b01486

Chen, Yi., & Tan, T. (1998). "Inhibition of the c-Jun N-Terminal Kinase (JNK) Signaling Pathway by Curcumin." *Oncogene*, 17(2): 173–178. doi: 10.1038/sj.onc.1201941

Dhillon, N., Aggrawal, B., Newman, R., Wolff, R., Kunnumakkara, A., Abbruzzese, J., Chaan, S., Badmaev, V., & Kurzroxk, R. (2008). "Phase II Trial of Curcumin in Patients with Advanced Pancreatic Cancer." *Clinical Cancer Research*, 14(14): 4491–4499. doi: 10.1158/1078-0432.CCR-08-0024

Dikshit, P., Goswami, A., Mishra, A., Chatterjee, M., & Jana, N. (2006). "Curcumin Induces Stress Response, Neurite Outgrowth and Prevent NF-kappaB by Inhibiting the Proteosome Function." *Neurotox Res*, 9(1): 29–37.

Ding, L., Ma, S., Lou, H., Sun, L., & Ji, M. (2015). "Synthesis and Biological Evaluation of Curcumin Derivatives with Water-Soluble Groups as Potential Antitumor Agents : An in Vitro Investigation Using Tumor Cell Lines." *Molecules* 20(12): 21501–21514. doi: 10.3390/molecules201219772

Divya, C., & Pillai, M. (2006). "Antitumor Action of Curcumin in Human Papillomavirus Associated Cells Involves Downregulaton of Viral Oncogenes, Prevention of NFkB and AP-1 Translocation and Modulation of Apoptosis." *Molecular carcinogenesis*, 45(5): 320-332. doi: 10.1002/mc.20170

Dua, J., Rana, A., & and Bhandari, A. (2012). "Liposome : Methods of Preparation and Applications." *International Journal of Pharmaceutical Studies and Research*, 3(2): 14–20. doi: 10.1016/j.ejpb.2013.01.028

Edgar, J. (2016). "Q & A: What Are Exosomes, Exactly?" *BMC Biology*, 14(1): 1–7. doi: 10.1186/s12915-016-0268-z

Eibl, H. (1980). "Synthesis of Glycerophospholipids." *Chemistry and Physics of Lipids*, 26(4): 405–429. doi: 10.1016/0009-3084(80)90061-4

Eklund, K., Vjrtanen, J., & Kjnnunen, P. (1984). "Involvement of Phospholipid Stereoconfiguration in the Pretransition of Dimyristoylphosphatidylcholine." *Biochimica et Biophysica Acta (BBA)*, 793(1): 310–312. doi: 10.1016/0005-2760(84)90334-5

El Khoury, E., Abiad, M., Kassaify, Z., & Patra, D. (2015). "Green Synthesis of Curcumin Conjugated Nanosilver for the Applications in Nucleic Acid Sensing and Anti-Bacterial Activity." *Colloids and Surfaces B: Biointerfaces*, 127: 274–280. doi: 10.1016/j.colsurfb.2015.01.050

El Khoury, E., & Patra, D. (2013). "Ionic Liquid Expedites Partition of Curcumin into Solid Gel Phase but Discourages Partition into Liquid Crystalline Phase of 1,2-Dimyristoyl-Sn- Glycero-3-Phosphocholine Liposomes." *Journal of Physical Chemistry B*, 117(33): 9699–9708. doi: 10.1021/jp4061413

El Khoury, E., & Patra, D. (2016). "Length of Hydrocarbon Chain Influences Location of Curcumin in Liposomes: Curcumin as a Molecular Probe to Study Ethanol Induced Interdigitation of Liposomes." *Journal of Photochemistry and Photobiology B: Biology*, 158: 49–54. doi: 10.1016/j.jphotobiol.2016.02.022

Eustis, S., & El-Sayed, M. (2006). "Why Gold Nanoparticles Are More Precious than Pretty Gold: Noble Metal Surface Plasmon Resonance and Its Enhancement of the Radiative and Nonradiative Properties of Nanocrystals of Different Shapes." *Chem. Soc. Rev.*, 35(3): 209–217. doi: 10.1039/B514191E

Fong, K., & Yung, L. (2013). "Localized Surface Plasmon Resonance: A Unique Property of Plasmonic Nanoparticles for Nucleic Acid Detection." *Nanoscale*, 5(24): 12043–12071. doi: 10.1039/c3nr02257a

Garg, S., & Garg, A. (2018). "Encapsulation of Curcumin in Silver Nanoparticle for Enhancement of Anticancer Drug Delivery." *International Journal of Pharmaceutical Sciences and Research*, 9(3): 1160–1166. doi: 10.13040/IJPSR.0975-8232.9(3).1160-66

Giacinti, C., & Giordano, A. (2006). "RB and Cell Cycle Progression." *Oncogene*, 25(38): 5220–5227. doi: 10.1038/sj.onc.1209615

Gliga, A., Skoglund, S., Wallinder, I., Fadeel, B., & Karlsson, H. (2014). "Size-Dependent Cytotoxicity of Silver Nanoparticles in Human Lung Cells: The Role of Cellular Uptake, Agglomeration and Ag Release." *Particle and Fibre Toxicology*, 11(1): 1–17. doi: 10.1186/1743-8977-11-11

Goel, A., Kunnumakkara, A., & Aggarwal, B. (2008). "Curcumin as 'Curecumin': From Kitchen to Clinic." *Biochemical Pharmacology*, 75(4): 787–809. doi: 10.1016/j.bcp.2007.08.016

Gregoriadis, G., Leathwood, P., & Ryman, B. (1971). "Enzyme Entrapment in Liposomes." *FEBS Letters*, 14(2): 95–99. doi: 10.1016/0014-5793(71)80109-6

Gunes, H., Gulen, D., Multu, R., Gumus, A., Tas, T., & Topkaya, A. (2016). "Antibacterial Effects of Curcumin: An in Vitro Minimum Inhibitory Concentration Study." *Toxicology and Industrial Health*, 32(2): 246–250. doi: 10.1177/0748233713498458

Guo, D., Xie, G., & Luo, J. (2014). "Mechanical Properties of Nanoparticles: Basics and Applications." *Journal of Physics D: Applied Physics*, 47(1): 1–26. doi: 10.1088/0022-3727/47/1/013001

Gupta, S., Patchva, S., Koh, W., & Aggarwal, B. (2012). "Discovery of Curcumin, a Component of Golden Spice, and Its Miraculous Biological Activities." *Clinical and Experimental Pharmacology and Physiology*, 39(3): 283–299. doi: 10.1111/j.1681.2011.05648.x

Heberle, F., & Feigenson, G. (2011). "Phase Separation in Lipid Membranes." *Cold Spring Harb Respect Biol*, 3(4)1–13. doi: 10.1101/cshperspect.a004630

Hoogevest, P., & Wendel, A. (2014). "The Use of Natural and Synthetic Phospholipids as Pharmaceutical Excipients." *European Journal of Lipid Science and Technology*, 116(9): 1088–1107. doi: 10.1002/ejlt.201400219

Huang, Z., & Haugland, R. (1991). "Partition Coefficinets of Fluorescents Probes with Phospholipids Membranes." *Biochemical and biophysical research communications*, 181(1): 166–171. doi: 10.1016/S0006-291X(05)81396-8

Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., & Thun, M. (2007). "Cancer Statistics, 2007." *CA: A Cancer Journal for Clinicians*, 57(1): 43–66. doi: 10.3322/canjclin.57.1.43

Karin, M., Liu, Z., & Zandi, E. (1997). "AP-1 Function and Regulation." *Current Opinion in Cell Biology*, 9(2): 240–246. doi: 10.1016/S0955-0674(97)80068-3

Khopde, S., Priyadarsini, K., Palit, D., & Mukherjee, T. (2000). "Effect of Solvent on the Excited-State Photophysical Properties of Curcumin." *Photochemistry and Photobiology*, 72(5): 625–631. doi: 10.1562/0031-8655(2000)0720625EOSOTE2.0.CO2

Kocher, A., Schiborr, C., Behnam, D., & Frank, J. (2015). "The Oral Bioavailability of Curcuminoids in Healthy Humans Is Markedly Enhanced by Micellar Solubilisation but Not Further Improved by Simultaneous Ingestion of Sesamin, Ferulic Acid, Naringenin and Xanthohumol." *Journal of Functional Foods*, 14: 183–191. doi: 10.1016/j.jff.2015.01.045

Kumar, A., Dhawan, S., Hardegen, N., & Aggarwal, B. (1998). "Curcumin (Diferuloylmethane) Inhibition of Tumor Necrosis Factor (TNF)-Mediated Adhesion of Monocytes to Endothelial Cells by Suppression of Cell Surface Expression of Adhesion Molecules and of Nuclear Factor-kappaB Activation." *Biochemical pharmacology*, 55(6): 775–83. doi: 10.1016/S0006-2952(97)00557-1

Landage, S., Wasif, A., & Dhuppe, P. (2014). "Synthesis of Nanosilver Using Chemical Reduction Methods." *International Journal of Advanced Research in Engineering and Applied Sciences* 3(5): 14–22. doi: 10.1080/17458080.2016.1139196

Li, G., He, D., Qin, Y., Guan, B., Gao, S., Cui, Y., Yokyama, K., & Wang, L. (2012). "Fungus-Mediated Green Synthesis of Silver Nanoparticles Using Aspergillus Terreus." *International Journal of Molecular Sciences*, 13(1): 466–476. doi: 10.3390/ijms13010466

Li, J., Wang, X., Zhang, T., Wang, C., Huang, Z., Luo, X., & Deng, Y. (2014). "A Review on Phospholipids and Their Main Applications in Drug Delivery Systems." *Asian Journal of Pharmaceutical Sciences*, 10(2): 81–98. doi: 10.1016/j.ajps.2014.09.004

Li, L., Aggarwal, B., Shishodia, S., Abbruzzese, J, & Kurzrock, R. (2004). "Nuclear Factor-κB and IκB Are Constitutively Active in Human Pancreatic Cells, and Their down-Regulation by Curcumin (Diferuloylmethane) Is Associated with the Suppression of Proliferation and the Induction of Apoptosis." *Cancer*, 101(10): 2351–62. doi: 10.1002/cncr.20605

Liu, Q., Loo, W., Sze, S., & Tong, Y. (2009). "Curcumin Inhibits Cell Proliferation of MDA-MB-231 and BT-483 Breast Cancer Cells Mediated by down-Regulation of NFκB, cyclinD and MMP-1 Transcription." *Phytomedicine*, 16(10): 916–922. doi: 10.1016/j.phymed.2009.04.008.

Liu, W., Wu, Y., Wang, C., Li, H., Wang, T., Liao, C., Cui, L., Zhou, Q., Yan, B., & Jiang, G. (2010). "Impact of Silver Nanoparticles on Human Cells: Effect of Particle Size." *Nanotoxicology*, 4(3): 319–330. doi: 10.3109/17435390.2010.483745

Majeed, S., Bin Abdullah, M., Nanda, M., & Ansari, M. (2016). "In Vitro Study of the Antibacterial and Anticancer Activities of Silver Nanoparticles Synthesized from Penicillium Brevicompactum (MTCC-1999)." *Journal of Taibah University for Science*, 10(4): 614–620. doi: 10.1016/j.jtusci.2016.02.010

Milobedzka, J., Kostanecku, S., & Lampe, V. (1910). "Zur Kenntnis Des Curcumins." *Chemische Berichte*, 43: 2163–2170. doi: 10.1002/cber.191004302168

Mourato, A., Gadanho, M., Lino, A., & Tenreiro, R. (2011). "Biosynthesis of Crystalline Silver and Gold Nanoparticles by Extremophilic Yeasts." *Bioinorganic Chemistry and Applications*, 2011: 1–8. doi: 10.1155/2011/546074

Mukhopadhyay, A., Banerjee, S., Stafford, L., Xia, C., Liu, M., & Aggarwal, B. (2002). "Curcumin-Induced Suppression of Cell Proliferation Correlates with down-Regulation of Cyclin D1 Expression and CDK4-Mediated Retinoblastoma Protein Phosphorylation." *Oncogene*, 21(57): 8852–8861. doi: 10.1038/sj.onc.1206048

Nasri, H., Sahinfard, N., Rafieian, M., Rafieian, S., Shirzad, M., & Rafieian-kopaei, M. (2014). "Turmeric: A Spice with Multifunctional Medicinal Properties." *Journal of HerbMed Pharmacology Journal J HerbMed Pharmacol*, 3(1): 5–8.

Okegawa, T., Pong, R., Li, Y., & Hsieh, J. (2004). "The Role of Cell Adhesion Molecule in Cancer Progression and Its Application in Cancer Therapy." *Acta biochimica Polonica*, 51(2): 445–457. doi: 035001445

Ollivon, M., Lesieur, S., Grabielle-Madelmont, C., & Paternostre, M. (2000). "Vesicle Reconstitution from Lipid-Detergent Mixed Micelles." *Biochimica et Biophysica Acta - Biomembranes*, 1508(1): 34–50. doi: 10.1016/S0304-4157(00)00006-X

Pankhurst, Q., Connolly, J., Jones, S., & Dobson, J. (2003). "Applications of Magnetic Nanoparticles in Biomedicine." *Journal of Physics D: Applied Physics*, 36(13): 1–16.

Patra, D., & Barakat, C. (2011). "Synchronous Fluorescence Spectroscopic Study of Solvatochromic Curcumin Dye." *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 79(5): 1034–1041. doi: 10.1016/j.saa.2011.04.016.

Patra, D., El Khoury, E., Ahmadieh, D., Darwish, S., & Tafech, R. (2012). "Effect of Curcumin on Liposome: Curcumin as a Molecular Probe for Monitoring Interaction of Ionic Liquids with 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine Liposome." *Photochemistry and Photobiology*, 88(2): 317–327. doi: 10.1111/j.1751-1097.2011.01067.x.

Philip, S., & Kundu, G. (2003). "Osteopontin Induces Nuclear Factor κB-Mediated Promatrix Metalloproteinase-2 Activation through IκBα/IKK Signaling Pathways, and Curcumin (diferulolylmethane) down-Regulates These Pathways." *Journal of Biological Chemistry*, 278(16): 14487–14497. doi: 10.1074/jbc.M207309200

Prusty, B., & Das. B. (2005). "Constitutive Activation of Transcription Factor AP-1 in Cervical Cancer and Suppression of Human Papillomavirus (HPV) Transcription and AP-1 Activity in HeLa Cells by Curcumin." *International Journal of Cancer*, 113(6): 951–960. doi: 10.1002/ijc.20668

Toda, S., Miyase, T., Arichi, H., Tanizawa, H., & Takino, Y. (1985). "Natural Antioxidants. III. Antioxidative Components Isolated from Rhizome of Curcuma Longa L." *Chemical and pharmaceutical bulletin*, 33(4): 1725–1728.

Ramsewak, R., DeWitt, D, & Nair, M. (2000). "Cytotoxicity, Antioxidant and Anti-Inflammatory Activities of Curcumins I-III from Curcuma Longa." *Phytomedicine*, 7(4): 303–308. doi: 10.1016/S0944-7113(00)80048-3.

Reddy, A., & Lokesh, B. (1994). "Studies on the Inhibitory Effects of Curcumin and Eugenol on the Formation of Reactive Oxygen Species and the Oxidation of Ferrous Iron." *Molecular and Cellular Biochemistry*, 137(1): 1–8.

Riaz, K., Nagy, A., Brown, R., Zhang, Q., Malghan, S., & Goering, P. (2017). "Silver Nanoparticles: Significance of Physicochemical Properties and Assay Interference on the Interpretation of in Vitro Cytotoxicity Studies." *Toxicology in Vitro*, 38: 179–192. doi: 10.1016/j.tiv.2016.10.012.

Ries, L., Melbert, D., Krapcho, M., Mariotto, A., Miller, B., Feuer, E., Clegg, L., Horner, M., Howlader, N., Eisner, M., Reichman, M., & Edwards, B. (2007). "Cancer Statistics Review." *National Cancer Institute*, http://seer.cancer.gov/csr/1975_2004/.

Roldán, M., Pellegri, N., & Sanctis, O. (2013). "Electrochemical Method for Ag-PEG Nanoparticles Synthesis." *Journal of Nanoparticles*, 2013: 1–7. doi: 10.1155/2013/524150/.

Shameli, K., Ahmad, M., Zamanian, A., Sangpour, P., Shabanzadeh, P., Abdollahi, Y., & Zargar, M. (2012). "Green Biosynthesis of Silver Nanoparticles Using Curcuma Longa Tuber Powder." *International Journal of Nanomedicine*, 7: 5603–5610. doi: 10.2147/IJN.S36786

Shameli, K., Ahmad, M., Jazayeri, S., Shabanzadeh, P., Sangpour, P., Jahangirian, H., Gharayebi, Y. (2012). "Investigation of Antibacterial Properties Silver Nanoparticles Prepared via Green Method." *Chemistry Central Journal*, 6(1): 1–10. doi: 10.1186/1752-153X-6-73

Sharma, R., Euden, S., Platton, S., Cooke, D., Shafayat, A., Hewitt, H., Marczylo, T., Morgan, B., Hemingway, D., Plummer, S., Pirmohamed, M., Gescher, A., & Steward, W. (2004). "Phase I Clinical Trial of Oral Curcumin : Biomarkers of Systemic Activity and Compliance." *Clinical Cancer Research.*, 10(2): 6847–6854. doi: 10.1158/1078-0432.CCR-04-0744

Shim, G., Kim, M., Park, J., & and Oh, Y. (2013). "Application of Cationic Liposomes for Delivery of Nucleic Acids." *Asian Journal of Pharmaceutical Sciences*, 8(2): 120–128. doi: 10.1016/j.ajps.2013.07.009

Shishodia, S., Chaturvedi, M., Aggarwal, B. (2007). "Role of Curcumin in Cancer Therapy." *Current Problems in Cancer*, 31(4): 243–305. doi: 10.1016/j.currproblcancer.2007.04.001

Shivaji, S., Madhu, S., & Singh, S. (2011). "Extracellular Synthesis of Antibacterial Silver Nanoparticles Using Psychrophilic Bacteria." *Process Biochemistry*, 46(9): 1800–1807. doi: 10.1016/j.procbio.2011.06.008

Shoba, G., Joy, D., Joseph, T., Majeed, M., Rajendran, R., & Srinivas, P. (1998). "Influence of Piperine on the Harmacokinetics of Curcumin in Animals and Human Volunteers." *Planta Med*, 64(5): 353–356. doi: 10.1055/s-2006-957450

Singh, S., & Aggarwal, B. (1995). "Activation of Transcription Factor NF-kappaB Is Suppressed by Curcumin (Diferuloylmethane)." *Journal of Biological Chemistry*, 270(42): 24995–25000. doi: 10.1074/jbc.270.42.24995

Sotiriou, G., Teleki, A., Camenzind, A., Krumeich, F., Meyer, A., Panke, S., & Pratsinis, S. (2011). "Nanosilver on Nanostructured Silica: Antibacterial Activity and Ag Surface Area." *Chemical Engineering Journal*, 170(2-3): 547–554. doi: 10.1016/j.cej.2011.01.099

Stoehr, L., Gonzalez, E., Stampfl, A., Casals, E., Duschl, A., Puntes, V., & Oostingh, G. (2011). "Shape Matters: Effects of Silver Nanospheres and Wires on Human Alveolar Epithelial Cells." *Particle and Fibre Toxicology*, 8: 1–15. doi: 10.1186/1743-8977-8-36

Suresh, A., Pelletier, D., Wang, W., Morrell-Falvey, J., Gu, B., & Doktycz, M. (2012). "Cytotoxicity Induced by Engineered Silver Nanocrystallites Is Dependent on Surface Coatings and Cell Types." *Langmuir*, 28(5): 2727–2735. doi: 10.1021/la2042058

Takahashi, M., Uechi, S., Takara, K., Asikin, Y., & Wada, K. (2009). "Evaluation of an Oral Carrier System in Rats: Bioavailability and Antioxidant Properties of Liposome-Encapsulated Curcumin." *Journal of Agricultural and Food Chemistry*, 57(19): 9141–9146. doi: 10.1021/jf9013923

Thacker, P., & Karunagaran, D. (2015). "Curcumin and Emodin Down-Regulate TGF- B Signaling Pathway in Human Cervical Cancer." *PLoS ONE*, 10(3): 1–27. doi: 10.1371/journal.pone.0120045

Tien, D., Tseng, K., Liao, C., Huang, J., & Tsung, T. (2008). "Discovery of Ionic Silver in Silver Nanoparticle Suspension Fabricated by Arc Discharge Method." *Journal of Alloys and Compounds*, 463(1-2): 408–411. doi: 10.1016/j.jallcom.2007.09.048

Tiwari, G., Tiwari, R., Sriwastawa, B., Bhati, L., Pandey, S., Pandey, P., & Bannerjee, S. (2012). "Drug Delivery Systems: An Updated Review." *International Journal of Pharmaceutical Investigation*, 2(1): 1–11. doi: 10.4103/2230-973X.96920.

Torchilin, V. (2005). "Recent Advances with Liposomes as Pharmaceutical Carriers." *Nature Reviews Drug Discovery*, 4(2): 145–160. doi: 10.1038/nrd1632

Tyagi, P., Singh, M., Kumari, H., Kumari, A., & Mukhopadhyay, K. (2015). "Bactericidal Activity of Curcumin I Is Associated with Damaging of Bacterial Membrane." *PLoS ONE*, 10(3): 1–15. doi: 10.1371/journal.pone.0121313

Vasanth, K., Ilango, K., MohanKumar, R., Agrawal, A., & Dubey, G. (2014). "Anticancer Activity of Moringa Oleifera Mediated Silver Nanoparticles on Human Cervical Carcinoma Cells by Apoptosis Induction." *Colloids and Surfaces B: Biointerfaces*, 117: 354–359. doi: 10.1016/j.colsurfb.2014.02.052

Verma, A., Jain, N., Singha, S., Quraishi, M., & Sinha, I. (2016). "Green Synthesis and Catalytic Application of Curcumin Stabilized Silver Nanoparticles." *J Chem Sci*, 128(12): 1871–1878. doi: 10.1007/s12039-016-1189-7

Viswanathan, M., Arumugam, S., & Thangavel, B. (2016). "In Vitro Anticancer Activity of Silver Nanoparticle Synthesized by Escherichia Coli VM1 Isolated from Marine

Sediments of Ennore Southeast Coast of India." *Enzyme and Microbial Technology*, 95: 146–154. doi: 10.1016/j.enzmictec.2016.09.008

Vogel, & Pelletier, J. (1815). "Examen Chimique de La Racine de Curcuma." *Journal of Photochemistry*: 289–300.

Wang, C., Mayo, M., & Baldwin, A. (1996). "TNF- and Cancer Therapy-Induced Apoptosis : Potentiation by Inhibition of NF-κB." *American Association for the Advancement of Science*, 274(5288): 784–787. doi: 10,1126/science.274.5288.784

Wei, L., Lu, J., Xu, H., Patel, A., Chen, Z., & Chen, G. (2015). "Silver Nanoparticles: Synthesis, Properties, and Therapeutic Applications." *Drug Discovery Today*, 20(5): 595–601. doi: 10.1016/j.drudis.2014.11.014

Widakowich, C., Azambuja, E., Gil, T., Cardoso, F., Dinh, P., Awada, A., & Piccart-Gehbart, M. (2007). "Molecular Targeted Therapies in Breast Cancer: Where Are We Now?" *International Journal of Biochemistry and Cell Biology*, 39(7-8): 1375–1387. doi: 10.1016/j.biocel.2007.04.015

Wise, R., Hart, T., Cars, O., Streulens, M., Helmuth, R., Huovinen, P., & Sprenger, M. (1998). "Antimicrobial Resistance. Is a major threath to public heath" *BMJ (Clinical research ed.)*, 317(7159): 609–610.

Xia, Y., Makris, C., Su, B., Li, E., Yang, J., Nemerow, G., & Karin, M. (2000). "MEK Kinase 1 Is Critically Required for c-Jun N-Terminal Kinase Activation by Proinflammatory Stimuli and Growth Factor-Induced Cell Migration." *Proceedings of the National Academy of Sciences*, 97(10): 5243–5248. doi: 10.1073/pnas.97.10.5243

Xia, Z., Ma, Q., Li, S., Zhang, D., Cong, L., Tian, Y., & Yang, R., (2016). "The Antifungal Effect of Silver Nanoparticles on Trichosporon Asahii." *Journal of Microbiology, Immunology and Infection*, 49(2): 182–188. doi: 10.1016/j.jmii.2014.04.013

Xu, W., Ling, P., Zhang, T. (2013). "Polymeric Micelles, a Promising Drug Delivery System to Enhance Bioavailability of Poorly Water-Soluble Drugs." *Journal of Drug Delivery*, 2013(1): 1-15. doi: 10.1155/2013/340315

Yang, K., Lin, L., Tseng, T., Wang, S., & Tsai, T. (2007). "Oral Bioavailability of Curcumin in Rat and the Herbal Analysis from Curcuma Longa by LC-MS/MS." *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 853(1-2): 183–189. doi: 10.1016/j.chromb.2007.03.010

Yoysungnoen-Chintana, P., Bhattarakosol, P., & Patumraj, S. (2014). "Antitumor and Antiangiogenic Activities of Curcumin in Cervical Cancer Xenografts in Nude Mice." *BioMed Research International*, 2014: 1-12. doi: 10.1155/2014/817972

Zhang, T., Wang, L., Chen, Q. & Chen, C. (2014). “Cytotoxic Potential of Silver Nanoparticles.” *Yonsei Medical Journal*, 55(2): 283–291. doi: 10.3349/ymj.2014.55.2.283