AMERICAN UNIVERSITY OF BEIRUT

FORMATION OF LIPOSOME MEDIATED POLY (DIALLYLDIMETHYLAMMONIUM CHLORIDE) BASED NANOPARTICLES TOWARDS BIOMEDICAL APPLICATION

by
ALAA KHALIL OTHMAN

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AN ABSTRACT OF THE THESIS OF

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Major: Chemistry

Title: Formation of Liposome Mediated poly (diallyldimethyl ammonium chloride) Based Nanoparticles Towards Biomedical Application

Nanosystems with various compositions and biological properties are being extensively investigated for drug and gene delivery applications. Many nanotechnology methods use novel nanocarriers, such as liposome, in therapeutic technology targeting drug delivery system. Liposomes are considered as extraordinary drug delivery system where they form vesicular structures consisting of bilayers, resulting from the dispersion of phospholipids in water. However, liposomes alone during drug delivery are known to suffer from drug leakage and instability. Therefore, the surface modification of the liposomes by coating or adding polymer has shown progress in their application in drug delivery.

In this presented work, liposomes were synthesized according to the thin film hydration method. The drug efficiency of the liposome was established according to the release of curcumin from the liposomes. Further, we investigated the drug release efficiency of two different types of phospholipids liposomes; 1, 2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and mixture of 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and DMPC liposome. Three different nanocapsules were prepared using poly(diallyldimethylammonium)chloride (PDAA) polymer and silica nanoparticles, in order to assure complete encapsulation of curcumin into the core of the liposome. For liposomes, which were formed by mixture of DMPC and DPPC, we synthesized three different ratios of mixture. The prepared mixtures were modified by one layer of the cationic polymer (PDDA).

Drug delivery profile was carried out for DMPC and mixture nanocapsules at three different pHs (4, 6 and 7) to test the release of curcumin. It was shown, at pH 4, curcumin exhibits higher release. Moreover, it was confirmed that when increasing the number of layers on the nanocapsule surface, curcumin exhibits lower curcumin release during 24 hours. Florescence, TGA, DLS, XRD, SEM, Zeta potential, encapsulation efficiency and drug loading were investigated for the different DMPC nanocapsules. Results show better encapsulation with the increase of the layer added, referring to the electrostatic interaction that inhibit curcumin from being released.

Mixture liposomes were also investigated by UV-visible Spectroscopy, Fluorescence, Zeta-potential, DLS and SEM. The analysis demonstrated when higher concentration of DPPC is used

higher encapsulation of curcumin is obtained. This difference is assigned to the longer tail chain in DPPC liposome than DMPC.

In this study, we also show the synthesis route of DMPC nanocapsule towards ATP detection. The prepared nanocapsule was analyzed using UV-vis spectroscopy, Fluorescence, DLS, SEM, Zeta-potential, TGA and XRD. The detection of ATP was carried out by monitoring the change in the fluorescence emission while increasing the concentration of ATP in the tested sample. The fluorescence intensity increased by the increase of ATP concentration. The nanoprobe gives a linear correlation between the intensity and the concentration with of detection equals to 0.11 μM.

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ABREVIATIONS

AD: Alzheimer's disease
ATP: Adenosine Triphosphate
CTP: Cytosine Triphosphate
DADMAC: (Diallyl dimethyl ammonium chloride)
DDW: Doubled Distilled Water
DLS: Dynamic Light Scattering
DMEM: Dulbecco's Modified Eagle's medium
DMSO: Dimethyl sulfoxide
DMPC: 2-dimyristoyl-sn-glycero-3-phosphocholine
DPPC: 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine
DTPE: 1,2-bis(10,12-tricosadiynoyl)-sn-glycero-3-phosphoethanolamine
EDX: Energy-Dispersive X-Ray
FBS: Fetal bovine serum
GTP: Guanine Triphosphate
HDL: High density-lipoprotein
IC_{50}: Half maximal inhibitory concentration
LOD: Limit of Detection
LUV: Unilamellar vesicles
lysoPC: Lyso-phosphatidylcholine
MLVs: Multilamellar vesicles
MTT: Thiazolyl Blue Tetrazolium Bromide
Si-MPs: Mesoporous silica nanoparticles
NMR: Nuclear Magnetic Resonance
MCF-7: Breast cancer cells.
N1: Nanocapsule 1
N2: Nanocapsule 2
N3: Nanocapsule 3
PAA: Poly(allylamine)
PBS: Phosphate Buffer Saline
PCL: Poly-e-caprolactone
PDAA: Poly(diallyldimethylammonium)chloride
PEI: Poly(ethyleneimine)
PIBCA: Poly(isobutylcyanoacrylate)
PLA: Poly lactide
PLGA: Poly(lactide-co-glicolide)

PSS: Poly(styrene sulfonate)
R.F.Q.Y: Relative flurescence quantum yield
ROS: reactive oxygen species
RRS: Resonance Rayleigh scattering
SEM: Scanning Electron Microscopy
SFS: Synchronous fluorescence spectroscopy
SPR: Surface Plasmon Resonance
SUV: Small unilamellar vesicles
TGA: Thermogravimetric analysis
TTP: Thymine Triphosphate
ULVs: Unilamellar vesicles
UTP: Uracil Triphosphate
XRD: X-Ray Diffraction

CHAPTER 1

INTRODUCTION

1.1. Nanocapsules

1.1.1. Background

A nanocapsule is made up of shell and a space in which desired substances or drug may be encapsulated,[1] and exists in the range between 10 nm to 1000 nm.[2] In general, it consists of a liquid/solid core in which the drug is placed into a cavity or layers, which is surrounded by a distinctive polymer membrane.[2] Recently, these species have received an important elaboration, particularly those prepared using biodegradable polymers, due to their significant use as site-specific drug delivery system.[3] However, these nanomaterials are pharmaceutically fascinating because of their oil-based central cavities. This distinct property will allow a high encapsulation level for lipophilic matter and thereby improves the drug delivery release.[4]

1.1.2. Preparation techniques of polymeric nanocapsules

The synthesis route of polymeric nanocapsules can be selected depending on the purposes, technological advantages, the properties of the substances entrapped and the polymeric materials to be used.[5,6] Hence, the different methods are classified in three categories: In the first method, the Nanocapsules are obtained by layer-by-layer assembly method. The second method consists in the polymerization of a monomer, which contains the emulsion polymerization and the interfacial polymerization method. The third method is polymerization of synthetic polymer including nano-precipitation and emulsion diffusion method.[7,8]

1.1.2.1. Layer-by-layer assembly

Layer-by-layer self-assembly is a robust and simple method; it is an approach to develop a thin film on solid support by exposing to positive and negative matters with spontaneous deposition of the oppositely charged ions to get polyelectrolyte capsules (Figure 1).[9] Layer-by-layer methods are found to have the higher encapsulation rate, about 80 %.[10] This method requires a colloidal template onto which is adsorbed a polymer layer, which will be washed. The solubility of polymer layer can be decreased by drop-wise addition of a miscible solvent. It is an essential tool for the preparation of multi component devices, allowing the assembly of different matters by electrostatic, dipole–dipole, hydrogen bonds, and van der Waals interactions. In these systems, one layer is responsible for the chemical and physical properties, while the other one is responsible for the stabilization of the structure.[11]

The most used polycations are polylysine, chitosan, gelatin-B, poly (allylamine) (PAA), poly(ethylene mine) (PEI), and protamine sulfate. The polyanions are sodium alginate, poly (styrene sulfonate) (PSS), poly (acrylic acid), dextran sulfate carboxy methylcellulose, haluronic acid, gelatin-A, chondroitin and heparin. [8]

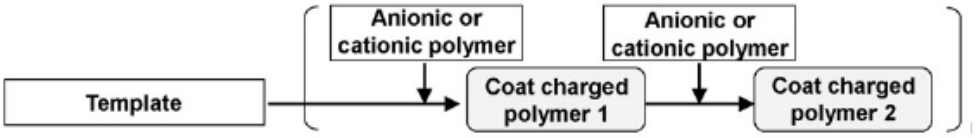

Figure 1: Schematic Illustration of layer by layer assembly technique.

In general, the capsules formed using layer-by-layer method is synthesized in the following steps:

1. Addition of the cationic or anionic polymer.

2. Layer by layer self-assembling to get an ultrathin polymer film. It is important to make sure that each new layer has the opposite charge compared to the previous layer added. The polymer coating is accomplished by electrostatic gravities.

3. Finally, shells of well-ordered polyelectrolyte complex layers are obtained.

1.1.2.2. Emulsion Polymerization

Emulsion polymerization is one of the fastest methods for nanocapsules preparation. Simply, the monomer is dissolved in an aqueous solution with the surfactants or emulsifiers. The polymerization process occurs when a monomer molecule dissolved in the continuous phase collides with an initiator molecule that might be an ion or a free radical (Figure 2). Alternatively, the monomer molecule can be transformed into an initiating radical by high-energy radiation strong visible light to get finally neutralized solution and the nanocapsules are filtered and present in suspension.

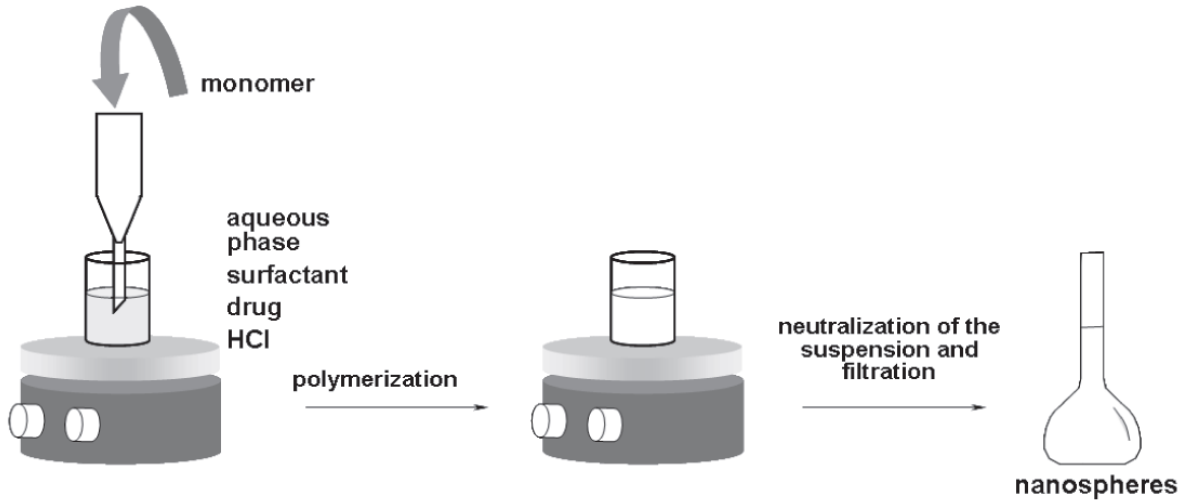

Figure 2: Scheme of emulsion polymerization technique.

1.1.2.3. Interfacial Polymerization

In this method, the monomer and the drug are mixed together in organic solvent. The nanocapsules are formed spontaneously by polymerization of the monomer after contact with initiating ions present in the water (Figure 3). The resulting colloidal suspension can be concentrated by evaporation under vacuum. The final product is a suspension of nanocapsules. PIBCA poly(isobutylcyanoacrylate) and poly(isohexylcyanoacrylate) are used in production of nanoparticles by this process in addition to the oil and the surfactant. An advantage of interfacial polymerization techniques is high-efficiency of drug encapsulation. In addition, the advantage of obtaining nanocapsules by this method is that the polymer is formed *in situ*, allowing the polymer membrane to follow the contours of the inner phase of an oil/water or water/oil emulsion.[7,12]

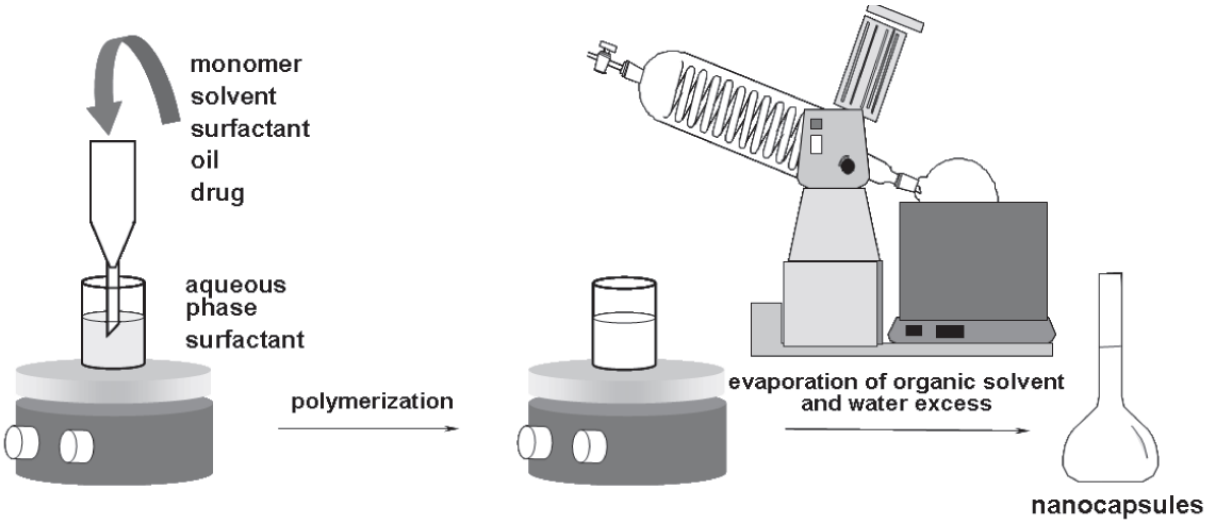

Figure 3: Scheme of interfacial polymerization technique.

1.1.2.4. Nano-precipitation Method

This method is also known as solvent displacement or interfacial deposition and classified in the physiochemical category.[13] Briefly, this method needs both solvent phase as

organic media which consists of a solution in a solvent or a mixture of solvent like ethanol, acetone, etc. and the non-solvent phase as water. This method necessities the use of biodegradable polymers, especially poly-e-caprolactone (PCL), poly lactide (PLA) and poly(lactide-co-glicolide) (PLGA), oils, surfactants and stabilizer agents. This method consists of adding the organic phase slowly to the aqueous phase with moderate stirring and after solvent evaporation the nanocapsules are formed in lipophilic and colloidal suspension (Figure 4). Hence, the nanocapsule core is composed by water/oil surfactants. However, the choice of the oil surfactant depends on the ability of the oil to increase the drug solubility and preventing the polymer degradation.[14,15]

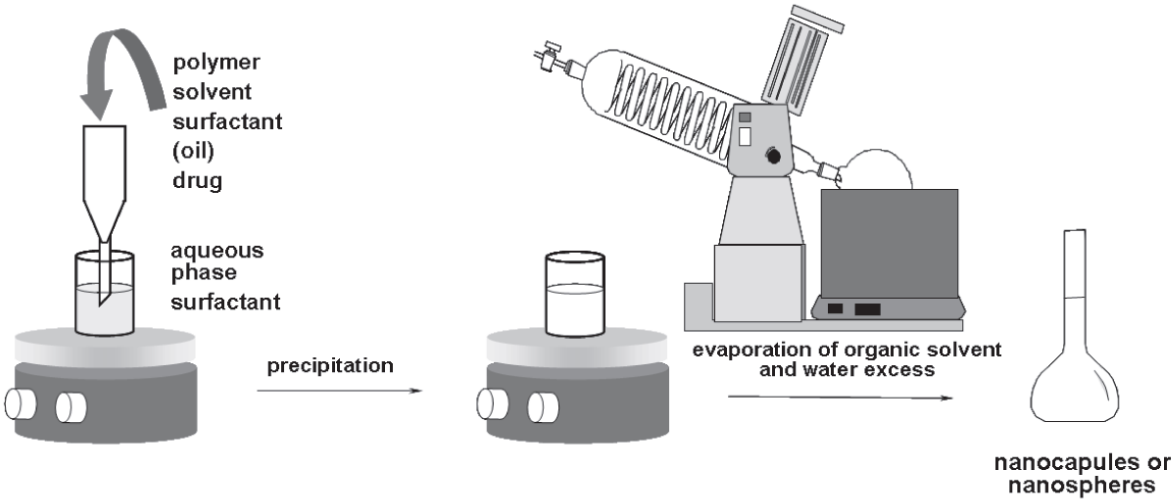

Figure 4: Scheme of nanoprecipitation method

1.1.2.5. Emulsion Diffusion Method

In this method, we can have both lipophilic and hydrophilic active substance for nanoencapsulation. Mainly, we need three phases; organic phase, aqueous phase and dilution phase. The aim of this method is the nanoencapsulation of a lipophilic active substance, oil and an organic solvent partially miscible with water. Thereby, the organic medium dissolves the

components of the organic phase, the inorganic phase can include oil as solvent and the aqueous phase comprises the aqueous dispersion of a stabilizing agent that is prepared using solvent-saturated water while the dilution phase is usually water (Figure 5). Polymers commonly used are biodegradable polyesters, especially PCL, PLA and a stabilizing agent is added when needed. The nanocapsules are finally formed after evaporation of the organic solvent and excess of water.[16,17]

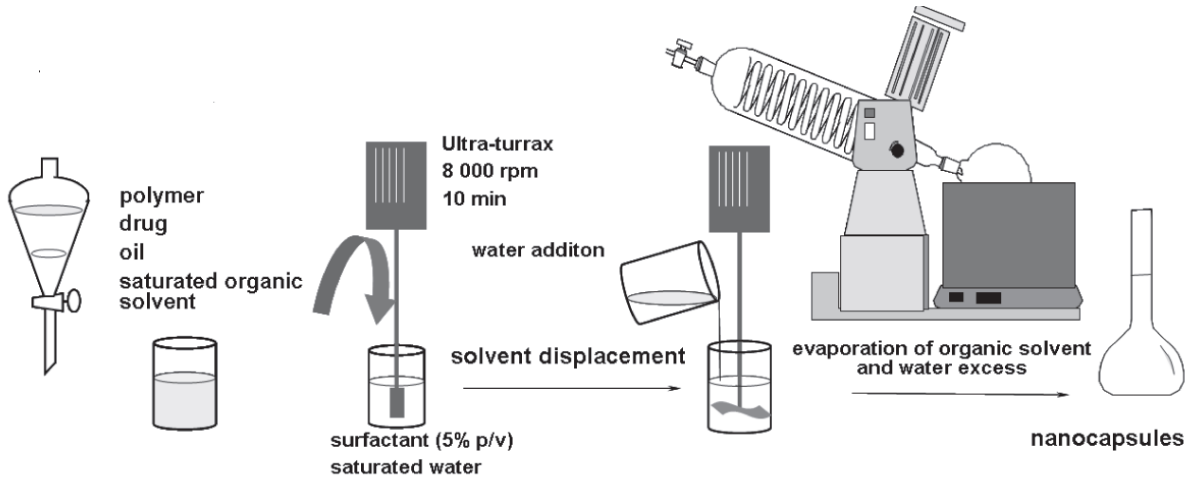

Figure 5: Scheme of emulsion diffusion technique

1.1.3. Composition of Polymeric Nanocapsules

The polymeric nanocapsules consist of two essential components: the liquid core of the nanocapsules and the surface which is the polymer membrane (Figure 6).

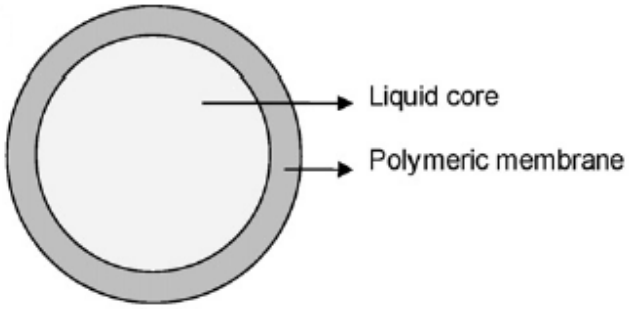

Figure 6: Components of liposomes

1.1.3.1. Core

The core in nanocapsules acts as a reservoir for the drug or active ingredient and can have biological activity or effect. One or more matter can be used as the structural material of the nanocapsules core. This specific structure of the core can dissolve or disperse easily the drug.[18]

1.1.3.2. Surface

Polymers have been used as the wall of nanocapsules. Thereby, the nanocapsule surface is directly related to the type of the polymer and the surfactants used. However, the production of nanocapsules using synthetic polymers induce higher reproducibility.[19]

1.1.4. Polymeric Nanocapsules in Drug Delivery

The binding of polymer with drug can be established by attaching the drug to the polymer chain by covalent linkage or by weak bonds as Van der Waals, hydrogen bonds, dipole-dipole bonds, etc.[20,21] This attachment will give three part in the chain starting with the solubilizing part responsible for the solubility of the drug, then we have the polymer-drug part where the drug is bound to the polymer chain and finally the transporting part that consists of the targeting molecules attached to the polymer chain.[21] Nanocapsules compared to the polymeric

nanospheres have high drug loading capacity as a consequence inducing high solubility of the drug in the core.[13] Nanoparticle surface is considered to have a great influence in studying drug release. Nanoparticles without surface modification and particles with negatively charged surfaces have been shown to be extensively cleared by macrophages in the blood stream. Thus, nano-drug release systems with positive surfaces are the best solution to protect the encapsulated drug from the rapid release and clearance.[22] Recently, many researchers have applied positively charged polymers, because of their simple, effective and facile strategy in holding the curative materials.[22]

Cationic polymers are positively charged molecules that have been generally utilized in various drug delivery systems. This refers to their ability in enhancing encapsulation efficacy and bioavailability. Additionally, studies on nanosystems based polymers have shown low toxicity and better drug release profile.[23]

Previous studies have shown that nanosized polymer-based particles have been tremendously tested to enhance the encapsulation efficiency and efficacy of the entrapped agents, as well as to control the release of the carried substances.[24] The nano-systems were clinically tested on tumor tissues, it was revealed that nanocarriers with 50~200 nm size are aggregated in the cancers area showing enhancement in time circulation, permeability and adequate drug release.[25] Further studies have confirmed the potent performance of polymeric nanocapsule such as, decreasing drug's toxicity, keeping drug away from the enzymatic degradation and reducing their unbeneficial release.[26] Nanocapsules for biomedical purposes are synthesized based on polyvinylpyrrolidone, polylactic- and polyglycolic acids, poly-ε-caprolactone and polyalkyl cyanoacrylates and poly(dimethyldiallylammonium chloride) (PDDA). PDDA is a cationic polyelectrolyte and commonly used in the preparation of polymeric

nanocapsules.[27] It is a synthetic polyelectrolyte polymer which belongs to quaternary ammonium group.PDDA was first established in the 1950s.[28]

PDDA possesses a backbone of cyclic units resulting from the special cyclopolymerization of (diallyl dimethyl ammonium chloride) DADMAC.[28] Additionally, the highly hydrophilic charged quaternary ammonium groups provide the polymer with a high water solubility and solution properties correspond to those of strong polyelectrolytes.[29] Additionally, PDDA is one of the polymers that exhibits much importance in biomedical applications especially in drug delivery since it is biocompatible, possess high molecular weight (which make it able to circulate in blood stream for certain time), be easily administered and the method to synthesize PDDA is simple and cheap.[20]

1.1.5. Advantages of Nanocapsules

Nanocapsules are found to have numerous advantages in the curative field. Hence, they have the potency to recover the solubility of lipophilic, poorly water-soluble compounds and to shelter the unstable molecules from the biological changes. Furthermore, drugs encapsulated within the core of nanocapsules show adequate release and good targeting.[30] Their subcellular size allows relatively higher intracellular uptake than other particulate systems. Nanocapsules are best described as effective substance carrier, since they can promote the stability of active substances and can be biocompatible with tissues and cells. These properties refer to their subcellular size which allows more intracellular intake than other carriers systems.[31,32] Other advantages of nanocapsules include high drug encapsulation efficiency due to controlled drug solubility in the core, little polymer collection compared to other nanocomposites systems such as nanospheres, drug shelter by polymeric layer against degradation by pH or light, etc.[7,33]

Furthermore, recent development in nanotechnology and its conjunction with medicine and biology has generated nanoparticles to be applied for bimolecular detection.[34,35] Novel studies have shown how efficiently nanocapsules can chemically interact with biomolecules like (peptides, proteins and nucleic acids).[36,37] It was found that the high chemical reactivity of nanocapsules with biomolecules refers to their nanometer size.[38]

1.2. Liposomes

Lipids have the ability to self-assemble in aqueous media and form bilayers, micelles, and liposomes. Liposomes are small artificial vesicles of spherical shape that can be created from cholesterol and natural non-toxic phospholipids surrounding aqueous components.[39,40] Liposomes were first discovered in England in 1961 by Alec D. Bangham, who was studying phospholipids and blood clotting.[41]

1.2.1. Composition of liposomes

All lipids that contain phosphorus group are called phospholipids. However, phospholipids are surface-active amphiphilic molecules. They contain a polar head group and a lipophilic tail (Figure 7). The amphiphilic character of the phospholipids makes them suitable to use as emulsifier, wetting agent, solubilizer and liposome former. Phospholipids bilayers are the core structure of liposomes and cell membrane formation.[42] Phospholipids can either be natural like soybean, rapeseed, and sunflower seed or synthetic with defined specific molecular species of polar head groups or fatty acids. Examples on synthetic phospholipids are 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) saturated phospholipids used in liposomes formation and lipid bilayers to study biological

membranes.[43] Research has also indicated that DPPC-liposomes could serve effectively as a delivery vehicle for inducing immune responses against glycosphingolipid antigens [44]. Moreover, DMPC has proved its efficiency to be used in drug delivery system that aims to improve high density-lipoprotein (HDL) cholesterol level on rats.[45]

Recent studies have also shown the use of mixture of liposomes for different targets. [46,47] Buffo et. Al. have shown a method to predict the phase transition temperature of DPPC-DMPC mixture depending on Zeta-potential analysis at various liposome temperature.[47] Another study, by Sumit et. al. has illustrated the physiochemical characteristics of 1,2-bis(10,12-tricosadiynoyl)-sn-glycero-3-phosphoethanolamine (DTPE), DMPC and DPPC liposome mixture, using different methods like, Nuclear Magnetic Resonance.[46]

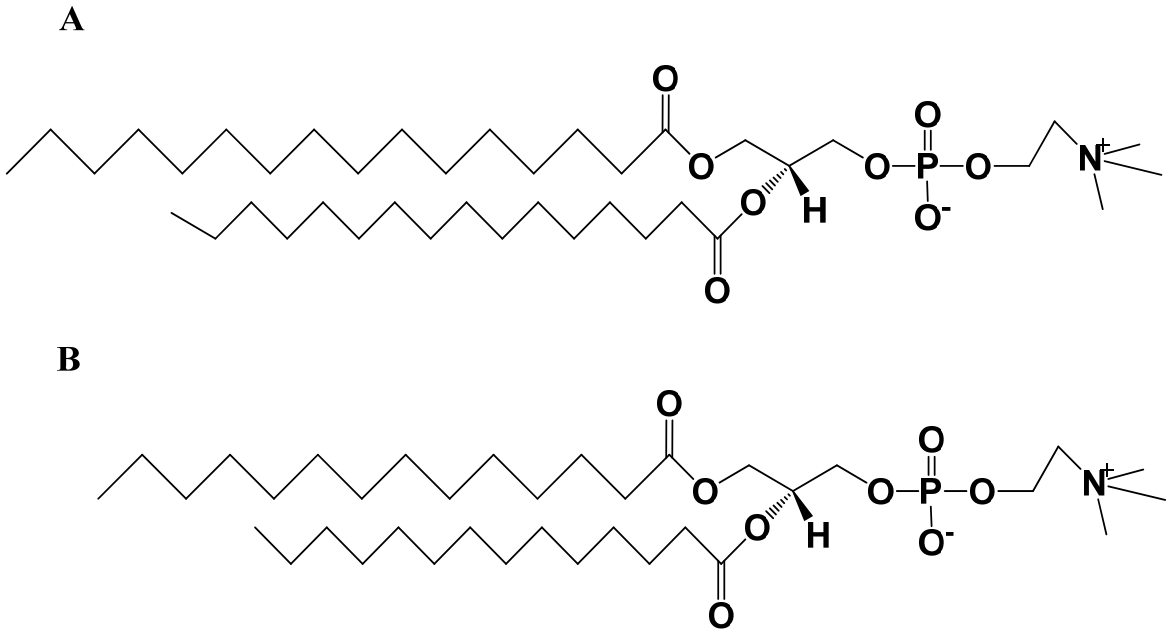

Figure 7: (A) Structure of DPPC and (B) structure of DMPC

1.2.2. Classification of Liposomes

Liposomes are classified according to the size and the number of membrane layers. The size of the liposomes could vary between 0.025 and 2.5 μm. Hence, these two parameter are essential in the amount of drug encapsulation in the liposomes and they lead to two categories of liposomes either multilamellar vesicles (MLVs) or unilamellar vesicles (ULVs) (Figure 8).[48] Multilamellar vesicles are liposomes that have a size greater than 0.1 μm.[49] Multilamellar vesicles have onion structure, it is obtained when several unilamellar vesicles will form one inside the other in smaller size, creating a concentric phospholipids spheres separated by layers of water.[50] Unilamellar vesicles are divided in two categories: large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV). In unilamellar liposomes, the vesicle has a single phospholipids bilayer sphere enclosing the aqueous solution less than 0.1 μm in size.[51,52]

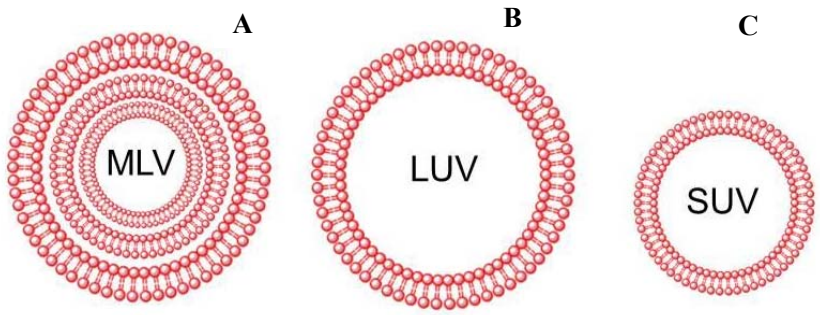

Figure 8: **Classification of liposomes; (A) multilamellar vesicles, (B) large unilamellar vesicles and (C) small unilamellar vesicles.**

1.2.3. Liposomes Surface Charge

The surface charge of the liposomes plays a key role in the *in vivo* disposition.[49] Neutral charge of liposomes leads to their aggregation and thereby it reduces the stability of the

liposomes. In consequence the interaction of neutrally charged liposome with the cell is negligible.[53] When the liposome surface is negatively charged the intracellular uptake of liposomes by target cells increases.[54] However, cationic liposomes deliver the contents to cells by fusion with cell membrane. Hence, they are preferably used in biomedicine application.[55]

1.2.4. Stability of Liposomes

During the synthesis of the liposomes, the physical and chemical stability of the developed formulation should be taken into consideration.

1.2.4.1. Physical stability

Once the liposomes are formed, during its storage, the vesicles tend to aggregate and induce an increasing in the size to attain thermodynamically favorable state.[56] However, the morphology and the size of the vesicles are important parameters to keep the physical stability. In order to control it, light scattering and electron microscopy techniques can be used to estimate the visual appearance and size of the vesicles.[42]

1.2.4.2. Chemical stability

Phospholipids are known as unsaturated fatty acids that submit oxidation and hydrolysis reaction, which can alter the stability of the drug product. In order to prevent this instability, pH, ionic strength, solvent system and buffered species play a major role in maintaining a liposomal formulation.[42] Liposomes can be prevented from oxidative degradation by protecting them from light. Lyso-phosphatidylcholine (lysoPC) can be formed after hydrolysis reaction of the ester group, which enhances the permeability of the liposomal contents and reduce the stability of the

liposomes. Hence, it is necessary to limit the production of lysoPC and this can be done by formulating liposomes with phosphatidylcholine free from lysoPC.[57]

1.2.5. Advantages of Liposomes

Liposomes are widely used as carriers for different molecules in cosmetics, therapeutic, food and pharmaceutical industries.[48] Basically, this refers to their tremendous advantages that were investigated in a wide range of studies and applications. Of these advantages, we can cite their ability to trap the hydrophobic and hydrophilic molecules without affecting their composition, where hydrophobic agents can be incorporated within the bilayer membrane and the hydrophilic agents can be inserted in the aqueous core.[58] Furthermore, liposomes release the entrapped matters to their specific target without affecting their composition.[39] Besides, these nanocarriers systems are considered as flexible, biocompatible, having the ability to form self-assembly and loading large molecules. Also liposomes offer a wide range of physicochemical and biophysical characteristics that can be adjusted to suit their biological environment.[58,59]

1.2.6. Applications of Liposome

Generally liposomes are well conducted to scientific, medicinal and pharmaceutical applications.[60] In biophysics, liposomes helped in the study of permeability, phase transitions in two-dimensions; in the chemistry field, it has evidence to study the photochemistry, artificial photosynthesis, catalysis, etc. In addition to the biochemistry domain where the reconstitution of membrane proteins into artificial membranes was developed, in biology the model biological membranes, cell function, fusion, recognition were investigated. As pharmaceutical application, researchers developed the studies of drug action and finally in the medicine sector the drug-

delivery, medical diagnostics and gene therapy, cancer and antimicrobial therapy were applied.[61] The liposomes have been found also to be useful in the respiratory and eye disorders, and brain targeting.[62]

1.2.7. Liposomes in Drug Delivery

Liposomes have shown their intense impact in drug delivery systems. Multi studies have been carried out on liposomes aiming to lesson drug toxicity and to target certain sites.[63,64] Hence liposomes have been shown to improve the stability, efficacy and therapeutic index of drug. Additionally, drug delivery based on liposomal encapsulation exhibited lower toxicity of the entrapped drugs, adequate targeting and flexibility in binding to specific agents to attain their targets. Further, liposomes work on reducing the contact between sensitive tissues and toxic drugs.[42] However, the use of liposomes in drug delivery has many benefits in the drug load. The use of liposomes increase the solubility of lipophilic and amphiphilic drugs through hydrophobic tails and aqueous core, improve the transfer of hydrophilic and charged molecules and enhance the penetration into tissues.[48]

To investigate the efficiency of liposome as a drug carrier, it is extremely important to test percent drug encapsulation. It shows how much of the drug is get encapsulated within the liposome.[42]

1.2.8. Preparation of Liposomes

Several liposomes preparation methods were developed in the literature. However, many parameters should be taken into consideration when choosing the preparation method as the physicochemical characteristics of the material to be entrapped and the concentration, the pH and

the nature of the medium, optimum size, poly dispersity and shelf-life of the vesicles for the intended application and batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products.[65]

In general, all the methods of liposomes preparation involve four main steps. First of all, the lipids are dried down from the organic solvent. Second, the lipids are dispersed in aqueous media. Third the formed liposomes are purified and finally the final product can be analyzed.[66] Passive loading techniques and active loading technique are used to prepare the liposomes. The passive loading technique include mechanical dispersion method, solvent dispersion method and detergent removal method.[40]

The mechanical dispersion methods contain different types:

- Sonication
- French pressure cell: extrusion
- Freeze-thawed liposomes
- Lipid film hydration by hand shaking, non-hand. shaking or freeze drying
- Micro-emulsification
- Membrane extrusion
- The solvent dispersion methods include:
- Ether injection (solvent vaporization)
- Ethanol injection
- Reverse phase evaporation method
- The detergent removal methods involve:
- Dialysis

- Detergent (cholate, alkyl glycoside, Triton X-100) removal of mixed micelles (absorption)
- Gel-permeation chromatography
- Dilution

1.3. Curcumin

1.3.1. Definition

Curcumin is a phytopolylphenol pigment derived from the rhizome *Curcuma longa*. *Curcuma longa* is a permmial herb that belongs to ginger.[67] It has been widely used as a spice, food-coloring agent and for medication in Asian countries many years ago. This refers to its antioxidant, anti-inflammatory, antimutagenic, antimicrobial and anticancer characteristics.[68]

1.3.2. Chemical properties

Curcumin or diferuloylmethane (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a gold color pigment with molecular mass of 368.385 g/mol and with molecular formula as $C_{21}H_{20}O_6$.Its melting point temperature is nearly 183 °C. It consists of two aryl rings that are composed of ortho-methoxy phenolic OH functional groups. Due to the existence of intermolecular hydrogen atom transfer (tautomerization mechanism) an equilibrium between the keto and the enol forms of curcumin exists (Figure 9).[69,70]

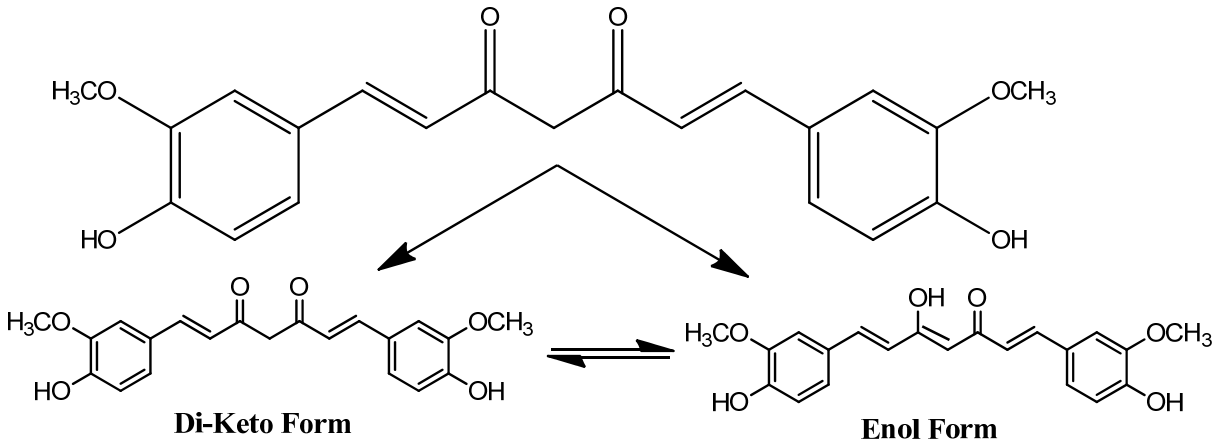

Figure 9*:* Tautomerization of curcumin.

Curcumin is considered as hydrophobic molecule, where its solubility is enhanced partially in basic solvents. Though, curcumin is highly soluble in organic solvents such as ethanol, acetone and dimethylsulfoxide.[71] Three curcuminioids compounds where found in the rhizome of Curcuma longa (See Figure 10). The main component complex was curcumin I with 77%, where curcumin II (demethoxycurcumin) with 17% and curcumin III(bis-demethoxycurcumin) with 3%.[72]

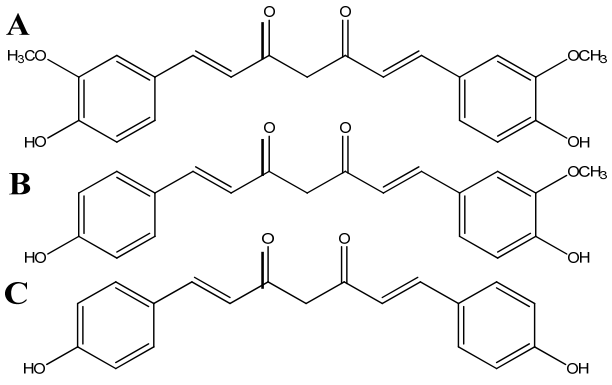

Figure 10: Different Type of Curcumin (A) Curcumin I, (B) Demethoxycurcumin (Curcumin II) and (C) Bis-demethoxycurcumin (Curcumin III).

1.3.3. Stability of Curcumin

Stability of curcumin is extremely important to conserve its physiological properties. Studies have revealed that under acidic and neutral conditions (pH 2.5–7.0), curcumin is insoluble and has bright yellow color. However, it changes into red under basic conditions (pH above 7) ,due to the deprotonation of OH functional groups making the molecule more polar and therefore more soluble. The dissociation of the three protons depends on pKa value as shown in figure 11.[73]

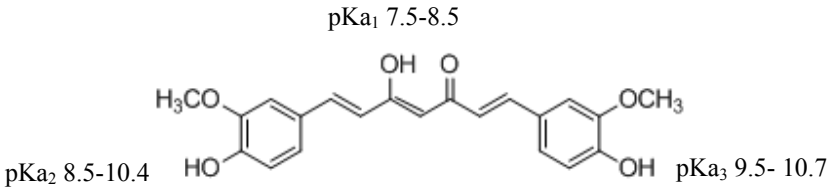

Figure 11: Dissociation of three protons of curcumin.

The degradation of curcumin relies on pH conditions, it was illustrated that curcumin decomposes faster under neutral-basic environments. However, it was found to be slower under acidic medium where about 20 % of curcumin decompose after 1 hour. The first pKa value is in this range 7.5-8.5, which results in the change of the solution from yellow to red color. The deprotonation of the other two protons depends on the pH value. The fully deprotonated curcumin exhibited maximum absorption at 467 nm at pH>10. [74]

1.3.4. Therapeutic use

Curcumin has been proven to have therapeutic impacts against multiple diseases, such as cancer, pulmonary diseases, neurological, liver, metabolic and cardiovascular diseases.[75]

Thus, extensive preclinical studies have specified the potent therapeutic impacts of curcumin towards numerous diseases. Thereby, it exhibits various pharmacological and biological functions as shown below.[76]

1.3.4.1. Antioxidant activity

Antioxidant activities are considered the major impacts of curcumin that explains its effectiveness.[77] It was shown that the oxidative bad results of DNA and protein are extremely linked with different chronic diseases such as cancer, neurodegenerative diseases, and aging.[78] Though curcumin is found to act as an antioxidant agent against oxidative-stress-mediated pathological conditions.

Peroxidation of lipids is defined to be a free-radical-mediated chain reaction. As a consequence, it results in damage to the cell membranes. Therefore, curcumin has shown a great inhibition of lipid peroxidation by removing the reactive free radicals, which are highly carcinogens.

Basically, this property of curcumin of being antioxidant agent, relies on its chemical structure, as it consists of β-diketo group, carbon–carbon double bonds, and phenyl rings containing varying amounts of hydroxyl and methoxy group.[79]

1.3.4.2. Anti-inflammatory activity

The inflammatory cells release a number of reactive forms in the inflammation region which result on oxidative stress. This attribution explains the link between oxidative stress and

inflammation.[80]

Thereby, inflammation was investigated to promote several diseases including Alzheimer's disease (AD), Parkinson's disease, cardiovascular disease, cancer, allergy, asthma, etc.[81,82] These diseases all are related to the cellular reactive oxygen species (ROS) collection and oxidative damage that occurs to lipids, nucleic acids and proteins.[72]

Curcumin has been shown to forbid inflammation through various mechanisms that ensure its potential as anti-inflammatory agent.[82]

1.3.4.3. Anti-cancer activity

Cancer's mechanism includes several steps that involve disruption of more than 500 genes at numerous stages in cell.[83] Subsequently, curcumin has shown several anti-cancer activities that can be used as a treatment against certain types of cancer such as colon, breast and lung cancers.[84]

The first therapeutic signal of curcumin against cancerous diseases was tested in 1987. It was indicated that curcumin, either alone or with cooperation with other drugs, has proved its effective action against cancer.[84] In addition, curcumin's anti-oxidant and free-radical potential suppress the development of carcinogens in the early phase of cancer diseases.[85] Subsequently, curcumin can either stop the growth of tumors, or it can result in apoptosis in cancer cells and can work on a several target molecules which aim to enhance cancer level of signal transduction pathways and molecular targets involved in the development of cancer.

1.3.5. Curcumin as sensor probe

Applying florescence methods in probing is extensively preferable, since they are simple, selective and sensitive compared to many established methods.[86] Though, curcumin as an organic fluorophore has been used by nanoparticles for detection targets.[87,88] Many studies were conducted to investigate the florescence profile of curcumin, as an example the work done by Khoury et. al.,[89] has proved the massive fluorescence ability of curcumin to find out some liposome's characteristics. Another study, by Terra et. al., have shown that curcumin with cellulose acetate complex, was used as colorimetric analytical technique for the detection of Pb(II).[88] Moreover, a study that was held by Patra et. al.,[90] have demonstrated DNA detection by the encapsulated curcumin based poly(diallylammonium chloride-co-sulfur dioxide) nanocapsule. Also, Bhat et. al. have reported the colorimetric method, using curcumin as a fluorescence probe for the detection of fluoride and iron ions.[87]

1.4. Aims

Nanotechnology has brought a tremendous effect in biomedical applications, especially in drug delivery. Moreover, nanomaterials have proved to increase the bioavailability and the solubility of numerous drugs. Curcumin has demonstrated its massive effect as anti-inflammatory, anti-oxidant and anticancer agent. On the other hand, curcumin lacks in solubility and bioavailability. Thereby, CHAPTER II of this work, is aimed to synthesize liposome based nanocapsule to enhance curcumin's biological properties. We formed PDDA based DMPC liposome for curcumin delivery. Three different cases of DMPC nanocapsule (N1, N2 and N3) were synthesized following layer-by layer self-assembly procedure. Hence, we were able to encapsulate curcumin in the core of the DMPC liposomes for each synthesized nanocapsule.

Then, the drug delivery profile was studied for N1, N2 and N3 at three different pHs (4, 6 and 7). After that, the synthesized nanocapsules were tested in breast cancerous cell to investigate the effect of curcumin from the released nanocapsules. Moreover, CHAPTER III of this study showed the formation of liposomal mixture consists of DPPC and DMPC liposomes, where curcumin was incorporated in the liposome core. Then, the formed mixture was coated with one layer of PDDA. The mixture was prepared in three different ratios. Afterwards, the drug release profile is reported for the three mixtures' ratios.

In general, curcumin as a florescence probe is well known to be used in sensing for various biomolecules. Hence, in CHAPTER IV of this study, we reported the encapsulation curcumin in DMPC based liposome coated with several PDDA layers, to detect ATP molecule. The encapsulated curcumin was set for different spectroscopic and morphological characterization. The crystallinity and thermal decomposition of the trapped curcumin was shown based on X-ray diffractogarm and thermogravemetric analysis (TGA) respectively. The emission fluorescence spectra of the nanocapsule were measured at different ATP concentrations. Furthermore, selectivity and stability study were performed to assess the specificity of the prepared nanocapsule for ATP sensing.

CHAPTER 2

DMPC LIPOSOMES BASED NANOCAPSULES FOR DRUG DELIVERY

2.1. Introduction

Recent chemotherapeutic techniques such as injection, oral tablets and intramuscular, have shown multi side effects on the growing normal cells with lower drug's accumulation in the target cells.[91,92] However, there was a significant need to find out new novel techniques in drug delivery field to target specific tumor cells.[93,94] Nanotechnology, has introduced tremendous studies focusing on the application of nanomaterial in medicine field, such as, drug delivery, gene delivery, tissue engineering, etc. Nanoscale drug delivery systems have interestingly proved, potent improvements in the biological properties of the drug including solubility and bioavailability.[95] Furthermore, drug delivery systems depending on nanomaterials have demonstrated early detection of tumor cells.[96]

Liposomes are microscopic materials that were extensively used for drug delivery targets.[97] Coating nanoparticles with cationic polymers such as PDDA, lower the toxicity of the encapsulated drug as well as improved its encapsulation.[26]

Curcumin is an important organic hydrophobic compound that has been used from ancient decades in food and as natural curative agent.[87] However, due to its low solubility and bioavailability, it is preferable to encapsulate it in nanoscale system such as liposomes.

Interestingly, curcumin is highly encapsulated in the lipid bilayer of liposome vesicles. In this CHAPTER, we reported the encapsulation of curcumin in DMPC liposome; the surface of the liposome is modified with different layers using PDDA and silica nanoparticles to form three

distinguished nanocapsules (N1, N2 and N3). Drug delivery profile is studied for the three different capsules of DMPC liposome. Moreover, cells study is conducted to examine the effect of the released curcumin from the capsules on the activity of breast tumor cells.

2.2. Materials and Methods

2.2.1. Materials

Curcumin, HS-40 Colloidal Silica, polydiallyldimethyl ammonium chloride (PDAA) and ethanol were obtained from Sigma-Aldrich.1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was obtained from Avanti. Chloroform was obtained from Sharlu. Buffer solutions (pH 4, 6, 7 and 10) were purchased from Fisher. All chemicals were used directly without further purification and were dissolved in double distilled water, expect for curcumin and DPPC, which were initially dissolved in ethanol and chloroform.

Dulbecco's Modified Eagle's medium (DMEM), penicillin/streptomycin were obtained from Lonza. Fetal bovine serum (FBS) was obtained from Sigma-Aldrich. Thiazolyl Blue Tetrazolium Bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Arcos Organics. PBS and trypsine were obtained from Sigma-Aldrich.

2.2.2. Synthesis of Liposomal Curcumin

The preparation of liposomal curcumin was done using thin film hydration method. Initially, 1.84 mg of curcumin was dissolved in chloroform and ethanol with 1:1 ratio (0.5 mL of ethanol and 0.5 mL of chloroform). Curcumin was added to 10mg of DMPC, which was dissolved in 2.5 mLof ethanol and 2.5 mL of chloroform (1:1 ratio). In a second step, the organic solvents were evaporated using rotary evaporator at 60 °C to accelerate the evaporation, where a thin film was obtained on the wall of the vial after complete evaporation. Afterwards, the sample

is kept in vacuum oven for 1 hour at 40 °C to eliminate all the traces of the organic solvents. Later on, 5 mL of buffer solution of pH 7 was added in order to dissolve the film. For this purpose, the solution was vigorously vortexed and heated for 10 minutes at 34 °C (10° C above the phase transition temperature of DMPC) in order to obtain a completely homogeneous solution with a hydrated thin film. Finally, to reduce the size of the liposomes, Mini-Extruder device from Avanti was utilized. The devise was applied for 20 times and the final solution of liposomal curcumin was ready to be used.

2.2.3. Preparation of Nanocapsules

Three different cases of nanocapsules were prepared as described below. In the first case, polymer layer was added to the liposomes surface. In the second case, silica nanoparticles were incorporated between two layers of polymer and finally in the third case, another layer of curcumin was added on the silica nanoparticles deposited in the second case. All these modifications were done using self-assembly procedure.

While preparing, all the solutions were centrifuged at 15, 000 rpm for 15 minutes using super speed centrifuge and all of the below illustrated cases were done each at three different pHs (4, 6, 7), where in the final step each sample was dissolved in different buffer solution pH 4, pH 6 or pH 7.Furthermore, the nanocapsules were prepared at alkaline condition pH= 10.However, the drug release at pH =10 is not included, since no biological applications were studied at strong alkaline medium.

2.2.3.1. Addition of the polymer layer at the liposome surfaces

In the beginning, 3 mL of the final liposomal curcumin solution were taken and mixed with 3 mL of 1 mg/mL of PDDA. Then the nanocapsule was kept in a water bath for 30 minutes at 34 ◦C followed by 30 minutes at room temperature. After that, the sample was centrifuged and 5 mL of buffer solution was added to the precipitate to get the nanocapsule. The nanocapsule N1 was composed of liposomal curcumin layer with an additional polymer layer, (Figure 12)

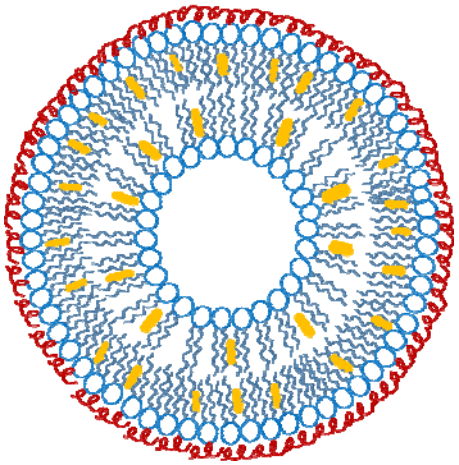

Figure 12: Schematic Illustration of N1 capsule.

2.2.3.2. Incorporation of silica nanoparticles

In this case, N1 solution was centrifuged and 5 mL of buffer solution was added to the solid layer followed by sonication. In a second step, 30 mL of LUDOX silica nanoparticles were added and the sample was kept for 1 hour at room temperature and centrifuged. After that, 3 mL of buffer solution and 3 ml of 1 mg/mL of PDDA solution were added to the sample and they all were properly mixed using sonication. Finally, the sample was kept for 30 minutes at 34 ◦C then for 30 minutes at room temperature and centrifuged to get the final nanocapsule N2 dissolved in 5 mL of buffer solution. The nanocapsule N2 was formed by the liposomal curcumin-PDDA-Silica-PDDA layer, (figure 13).

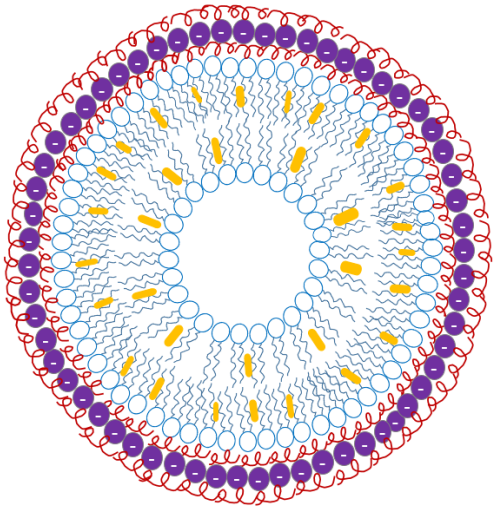

Figure 13: Schematic Illustration of N2 capsule.

2.2.3.3. Addition of curcumin

In this case, 50 µM of curcumin were added after the addition of silica nanoparticles (as it is described in III.2) and then centrifuged after 30 minutes. Later on, 3 mL of buffer solution and 3 mL of 1 mg/mL of PDDA were added and the solution was kept for 30 minutes at 34 ◦C and for 30 minutes at room temperature. Finally, the solution was centrifuged and 5 mL of buffer was added to the precipitate. In this case, the nanocapsules N3 consists of liposomal curcumin-PDDA-Silica-Curcumin-PDDA layer, (figure 14).

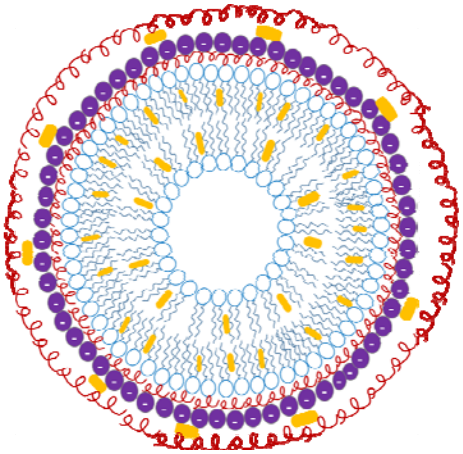

Figure 14: Schematic Illustration of N3 capsule.

2.2.4. Spectroscopic Measurements

The absorption spectra were recorded at room temperature using a JASCO V-570 UV–VIS–NIR spectrophotometer. The emission spectral measurements and synchronous fluorescence scan were documented with resolution increment 1 nm and slit 5 nm using Jobin-Yvon-Horiba Fluorolog III fluorometer and the FluorEssence program. The excitation source was a 100 W Xenon lamp, and the detector used was R-928 operating at a voltage of 950 V.

2.2.5. Drug loading and encapsulation efficiency

Drug loading and encapsulation efficiency was calculated based on measuring the absorbance of the released curcumin after each wash while preparing the three different DMPC nanocapsules. Later on, the final precipitate was dissolved in 1 mL DDW and dried in freeze dryer for 24 hours and the mass was measured.

2.2.6. Characterization techniques

Scanning electron microscopy (SEM) analysis was done using Tescan, Vega 3 LMU with Oxford EDX detector (Inca XmaW20). In short, few drops of nancapsules solution were deposited on an aluminum stub and coated with carbon conductive adhesive tape, and the sample was coated by Gold FTM. Zeta potential value was measured using Particulate systems, Nonplus Zeta Potential/Nano Particle analyzer.

2.2.7. Drug Delivery Release

After preparing the nanocapsules, the release of curcumin was studied at 37 °C according to the human body temperature. For this purpose, the nanocapsules were kept at 37 ◦C for 1 hour and then centrifuged. The absorbance of the supernatant was measured. As for the precipitate, 5 mL of buffer solution were added and kept for 1 hour at 37 °C and then centrifuged. This step was done at several times as the total hours for the whole curcumin release process was 24 hours, and the absorbance of the supernatant was always measured.

2.2.8. Culture of MCF-7 cancer cells

MCF-7 or breast cancer cells were cultured in a completed DMEM high glucose media, where 10% FBS, 1% penicillin/streptomycin were added to free DMEM high glucose. MCF-7 cells were cultured 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.

2.2.9. Cytotoxicity study by MTT proliferation Assay

After treating the cells with curcumin, silica, PDDA and the 3 different capsules; MTT assay was used to measure the cell activity. MCF-7 cells were seeded at a density of 5000 cells per well in 96-well plates. At 30% confluence, cells were subject to a concentration equal to 20 μM for the different treatment. After 72 hours, 1mg/mL of MTT was added to the cells and kept for 1 hour incubated at 37 °C. Later on the media with the MTT were eliminating from the 96-well plate and DMSO was added in order to solubilize the formazan crystals. ELISA microplate reader, Thermo/LabSystems 352 Multiskan MS, was used to read the plates at a wavelength of 595 nm.

2.3. Results and Discussion

2.3.1. Preparation of liposomal curcumin and the nanocapsules

Liposomal curcumin was prepared according to the thin film hydration method.[98] First, the phospholipids and the curcumin were dissolved in ethanol and chloroform with a ratio to 1:1. Second, the solvent was evaporated on a rotary evaporator at 60 °C until a uniform film appears, to end up with the addition of aqueous buffer which will lead to the hydration of the lipids and thereby the formation of the liposomal curcumin. The encapsulation of curcumin is completed in the hydrophobic part of the liposome. For the nanocapsules preparation, in the 3 different cases, a positively charged polymer layer was added on the liposome surface in a way to increase the stability of the liposomal curcumin, since liposomes have lack in their physical and chemical properties. In the first nanocapsule (N1), only one layer of PDAA was added to the liposome. For the 2nd nanocapsule (N2), one layer of negatively charged silica nanoaprticles was added to N1 and coated again with one layer of PDAA. Hence, silica nanoparticles were chosen to

maintain and reinforce the electrostatic interaction in the nanocapsules. Finally, for the 3rd nanocapsule (N3), curcumin was added just after the addition of silica nanoparticles, coated with a final layer of PDAA to assure more protection to the encapsulated curcumin.

2.3.2. Spectroscopic analysis for the different nanocapsules

Resonance Rayleigh Scattering, fluorescence emission and Uv-Visible spectra were recorded for N1, N2, N3 and free curcumin. Briefly, after centrifuging, the precipitate of the nanocapsules was dissolved in 5 mLof PBS buffer solution of pH equal to 7. As for curcumin, 1.84 mg of curcumin was dissolved in 3 mL of EtOH and PBS mixture (1:10) and the SFS intensity, emission intensity and absorbance were measured for each sample in 3mL cuvette.

Resonance Rayleigh Scattering (RRS), also known as Surface Plasmon Resonance (SPR), spectrum can be measured by applying synchronous fluorescence spectroscopy (SFS) by keeping the wavelength interval ($\Delta\lambda$) at 0 nm (See Figure 15A).[99] Synchronous flurescence spectrum for free curcumin exhibits two significat peakes at ~ 380 nm and ~ 501 nm. SFS spectra show an additional two peaks in the three nanocapsules nearly at 325 nm and 548 nm. In comparison with the SFS peaks of curcumin, a blue shift at ~370 nm and ~ 475 nm occurred in the tested nanocapsules. Thereby, the appearance of new peaks and the blue shift occurred assure the effect of PDDA layer surrounding the liposomal curcumin surface where the encapsulation of curcumin in the core of the liposomes is enhanced.

For the free curcumin and the three prepared nanocapsules of DMPC, the fluorescence emission spectra were measured at excitation wavelengths equal to and 425 nm and 350 nm in the emission range between 440-650 and 370-650, respectively. The results are shown in Figures 15B&C.

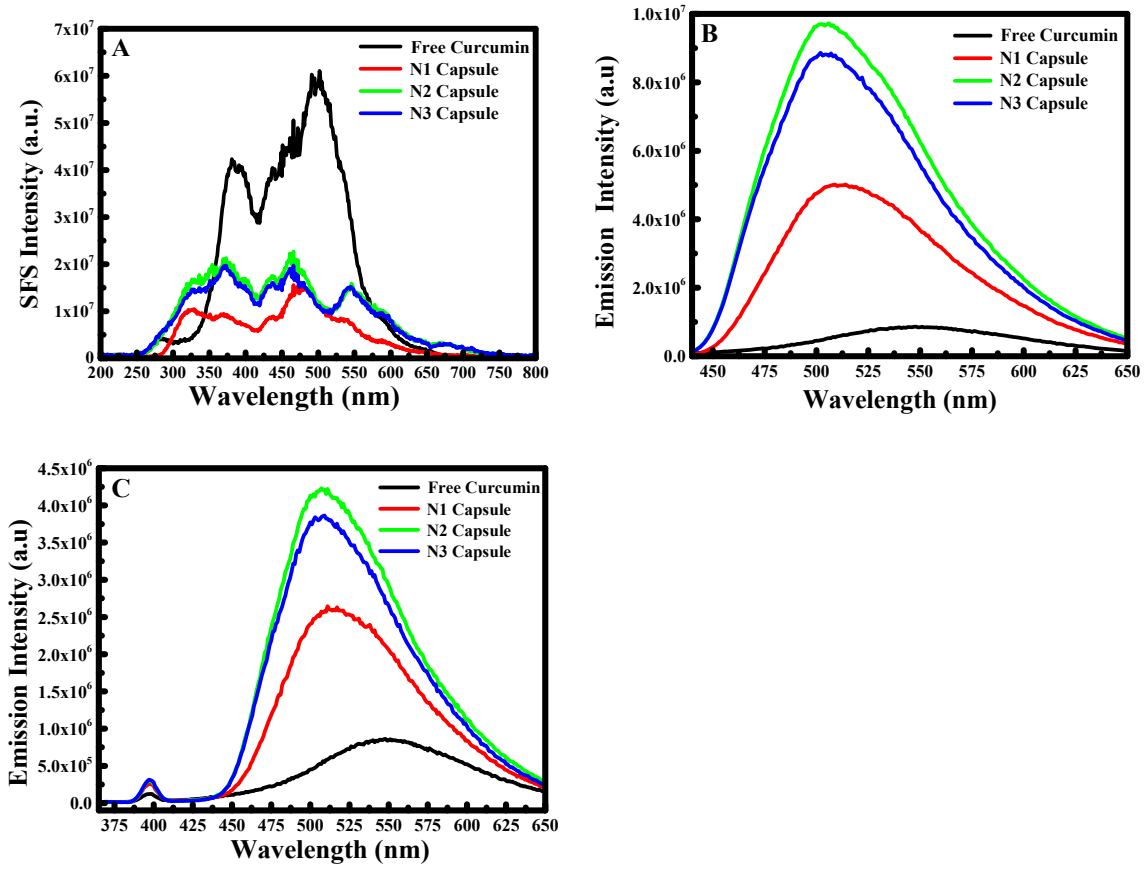

Figure 11: (A) Synchronous flurescence spectra at Δλ=0 nm, (B) Flurescence emission spectrum excited at λ = 425 nm and (C) Flurescence emission spectrum excited at λ = 350 nm for free curcumin, N1, N2 and N3.

The emission intensity of curcumin (initially dissolved in methanol), exhibits a major peak at λ~ 550 nm at both excitation wavelengths λ_{ex} = 425 nm and λ_{ex} = 350 nm. A blue shift to λ~ 510 nm occurred in 1st,2nd and 3rd capsules at λ_{ex} = 425 nm. Moreover, a blue shift to ~525nm was also noticed 1st ,2nd and 3rd type capsules at λ_{ex} = 350 nm. This blue shift is due to the polymer layer coating the surface of liposomal curcumin. In both emission intensities, N2 shows higher emission intensity. Thus, Relative Flurescence Quantum Yield was applied at both emission intensities at

425 nm and 350 nm. Relative flurescence quantum yield, is a specific property of the flurescent substances. Briefly,it is the ratio of the emission intensity to the aborbance of the flurescent substance at a definite wavelength, as it is shown in the equation 1.[100]

R.F.Q.Y = Emission Intensity / UV-vis Absorbance at specific wavelength (1)

The significance of ΦF has been investigated in many research studies such as estimation of audio visual equipment, dyes and fluorescent probes.[100] The relative flurescence quatum yields at λ = 425 nm and λ = 350 nm are shown in Figure 16 A&B.

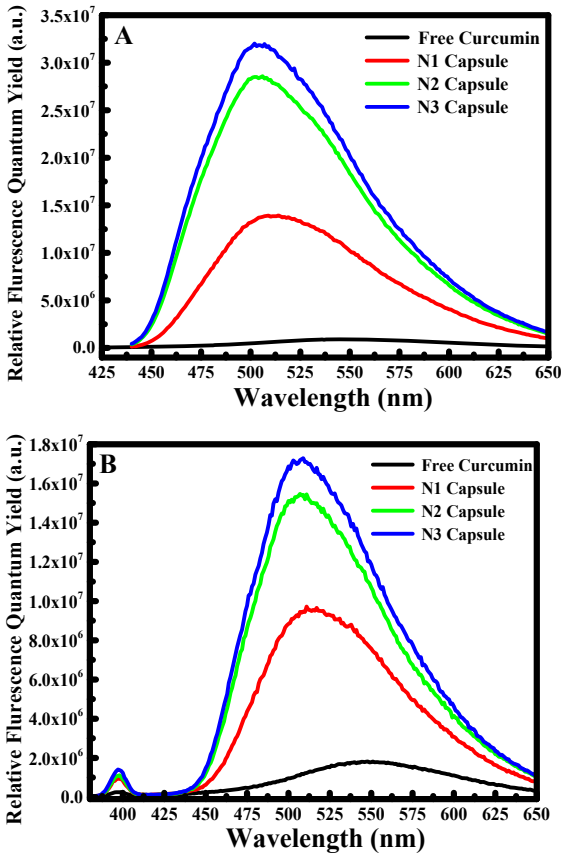

Figure 12: (A) Relative florescence quantum yield excited at λ = 425 nm and (B) Relative florescence quantum yield excited at λ = 350 nm for free curcumin, N1, N2 and N3.

It was found that the relative flurescence quantum yield enhances the flurescence intensity for the three nanocapsules. Hence, an additional enhancement was occurred for N1, N2 and N3 with high yeild for N3. However, at λ = 425 nm the increase can be estimated as 1.6, 2.8 and 3.5 folds for N1, N2 and N3 respectively. Additionally,at 350 nm,this enhancment can be estimated as 11.25, 5.76 and 4.59 folds for N1, N2 and N3 respectively. Thereby, the highest fluorescence emission yeild obtained for N3 verify the enhancement of the encapsulation of curcumin in N3.

Moreover, the absorbance of the three nanocapsules and curcumin was measured. The UV-Visible spectra are depicted in Figure 17. The characteristic peak of curcumin alone was found to be at λ= 425 nm, with a small red shift to λ= 428 nm for N1, N2 and N3. However, the relative absorbance for the 3 different capsules decreases for N3, N2 and N1, respectively compared to curcumin's absorbance. Thereby, for N3 where silica nanoparticles and curcumin layer were added, the obtained absorbance was ~ 0.36 a.u. greater than the absorbance of N2 and N1.

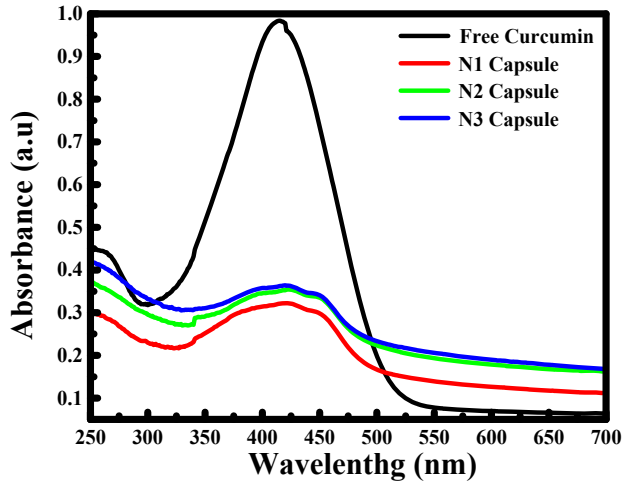

Figure 13: UV-Vis Spectra of free Curcumin, N1, N2 and N3.

Thus, curcumin is being more encapsulated in N3 and exhibits less release in the drug delivery profile opposite to the 1st and 2nd capsule. Furthermore, a ssharp peak was obtained for curcumin alone at λ= 425 nm. This peak tends to be more broad for the three nanocapsules, verifying the fact that curcumin is being encapsulated in the core of the liposomes and in correlation with the fluorescence data. According to Lambert-Beer's law, the absorbance is directly proportional to the concentration according to equation 2

$$A = l \times C \times \varepsilon \qquad (2)$$

Where A is the relative absorbance, l is the path length of the beam of light through the material sample, C is the concentration of the analyte and ε assigned to the molar extinction coefficient.The molar extinction coefficient was calculated based on the calibration curve of curcumin at pH 7, where ε was equal to the slope value. Hence, the concentration of curcumin was calculated for the 3 different nanocapsules and it was found to be equal to 15.01μM, 16.94 μM and 17.43μM for N1, N2 and N3 respectively.

2.3.3. Drug loading and encapsulation efficiency

The calculations of the concentrations and the masses of un-encapsulated curcumin were carried out according with the curcumin calibration curve at pH 7 (See Figure 18).

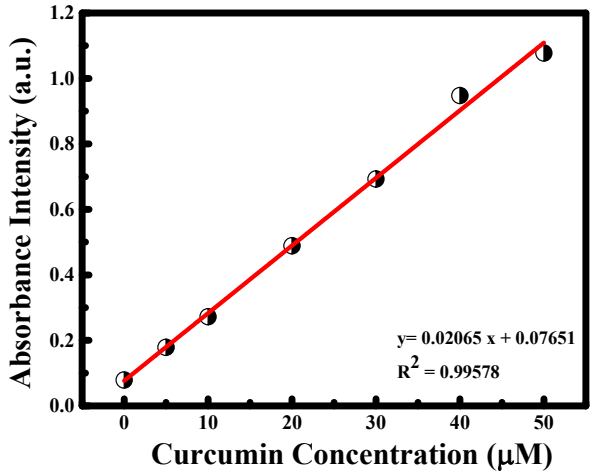

Figure 14: Calibration Curve of free Curcumin at pH 7.

The drug loading and encapsulation efficiency were established based on the below equations.[101]

$$\textbf{Drug loading \%} = \frac{Amount\ of\ curcumin\ in\ the\ capsule}{Total\ mass\ of\ the\ capsule}\ x\ 100$$

$$\textbf{Encapsulation Efficiency \%} = \frac{Amount\ of\ curcumin\ in\ the\ capsule}{Initial\ amount\ of\ curcumin}\ x\ 100$$

Where the amount of curcumin in capsule is equal to the amount of curcumin unencapsulated in the supernatent subtracted from the initial amount of curcumin.

The percentage of drug loading and encapsulation effeciency of N1, N2 and R3 are depicted in Table1.

Table 1: Relative percentage of Drug loading and encapsulation efficiency for N1, N2 and N3.

Nanocapsule	Drug loading %	Encapsulation efficiency %
N1	43.8	92
N2	22.7	87
N3	22.5	86

The calculations showed nearly same loading and encapsulation effeciency for N2 and N3. As for N1 92 % encapsulation efficiency was obtained. This highest value can be due to the fact that only one washing was occurred during the preparation of N1, although several washing were done while prepraring N2 and N3. Hence, less loss of curcumin was obtained in N1 compared to N2 and N3. The encapsulation efficiency results calculated in our experiment were similar to the value obtained by Lee et al. where the maximum encapsulation efficiency of curcumin was 93%[102] and higher compared to the value calculated by Young et al. that was equal to 80%.[103]

2.3.4. Characterization techniques

The morphology of the prepared nanocapsules was adopted using scanning electron microscopy (SEM). The nanoparticles of N1 formed by the self-assembly, show random and non-uniform spherical shapes (Figure 19A). However, after coating the surface of nanocapsule with additional layers as in N2 and N3, the nanoparticles aggregate and became more uniform and smaller than the nanoparticles obtained in N1 (Figure 19 B&C). Hence, the addition of extra

layer tends to force the penetration of the curcumin molecule into the core of the liposomes and therefor induce the formation of aggregation and smaller nanoparticles.

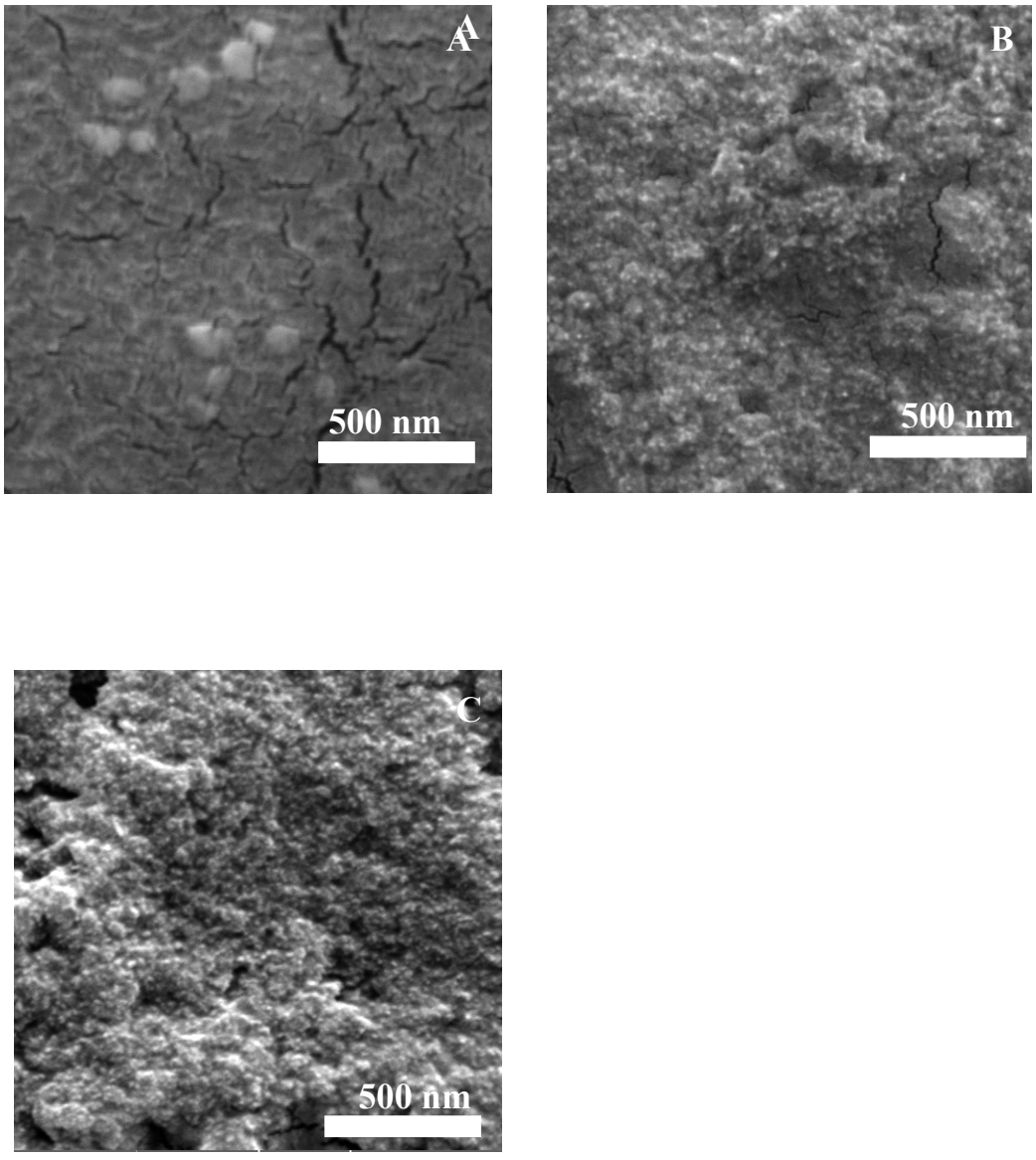

Figure 15: SEM images for (A) N1, (B) N2 and (C) N3.

Moreover, the particle size of the prepared nanocapsules was measured using Dynamic Light Scattering (DLS). Each sample was diluted and sonicated before size analysis was done. The results are shown in Figures 20 A, B&C.

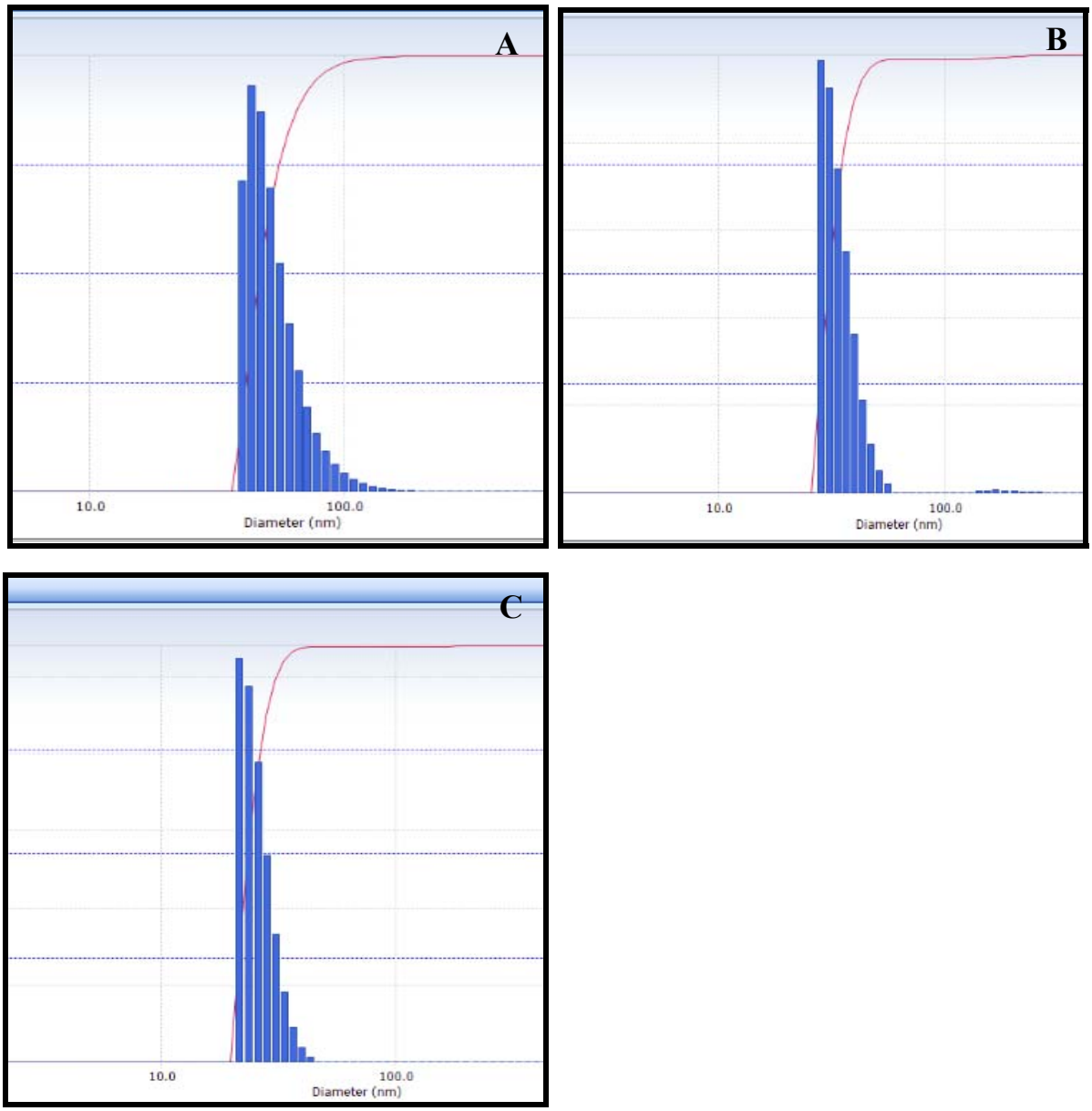

Figure 16: DLS measurements for (A) N1, (B) N2 and (C) N3.

The nanocapsules size was 207 nm, 297.2 nm and 240 nm for N1, N2 and N3 respectively. However, the nanocapsule size was approximatively around 200 nm and 300 nm. The difference in the three cases is around ± 40 nm, this change is due to the different aggregation occurred in the system and in the different layers added in the 3 cases. Although, DLS results verify the aggregation present in the solution, since SEM images have given nanoparticles size around 90 nm for N1 and 20-30 nm for N2 and N3.

To investigate the crystallinity of curcumin encapsulated in the three different DMPC capsules, X-ray diffraction (XRD) was performed. The diffractograms of free DMPC lipid, Free curcumin, N1, N2 and N3 are presented in Figure 21.

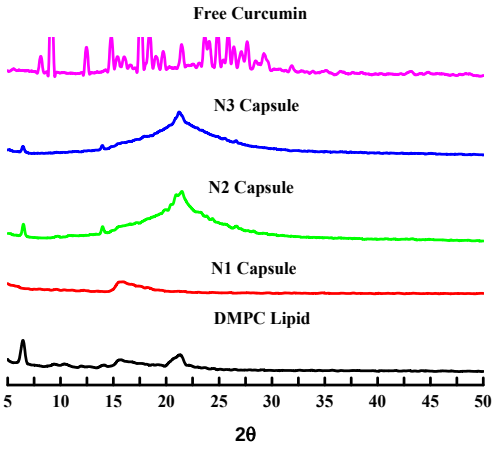

Figure 17: XRD Patterns of DMPC lipid, N1, N2, N3 and Free Curcumin.

The XRD spectra of the free DMPC lipid, illustrates the existence of three characteristic peaks at 6.4°, 15.7° and 21.3°, a number of minor peaks appeared in the 2Θ range of 9°-13.8°. The diffractogram of free curcumin shows significant peaks at 2Θ equal to 8.06°, 9.20°,12.46°,14.95°

,17.75° and some minor peaks existed with 2Θ range between 21.4° and 27.7° which implies that curcumin exhibits in the crystalline form.[104] On the other hand, none of capsules XRD patterns showed curcumin's characteristic peaks, expect 21.4° in N2 and N3 capsules. Moreover, the XRD patterns of N1, N2 and N3 are broad, as this suggests that the significant curcumin's XRD peaks became broader by the effect of the different coating layers on the surface of liposomal curcumin. This variety in the 2Θ angle can be assigned to the changes in the crystallinity structure of curcumin from crystalline to almost amorphous structure in the nanocapsules.[105,106]

To study the thermal properties of pure curcumin and for DMPC nanocapsules, Thermogarvemetric Analysis (TGA) was performed, as depicted in Figure 22. It is observed that no water loss occurred at 100 °C in N1, N2 and N3 capsules. Pure curcumin shows thermal decomposition between 240 °C – 560 °C.[99] In the other case, the obtained TGA patterns of the three different DMPC capsules demonstrated mass loss in the same temperature range.

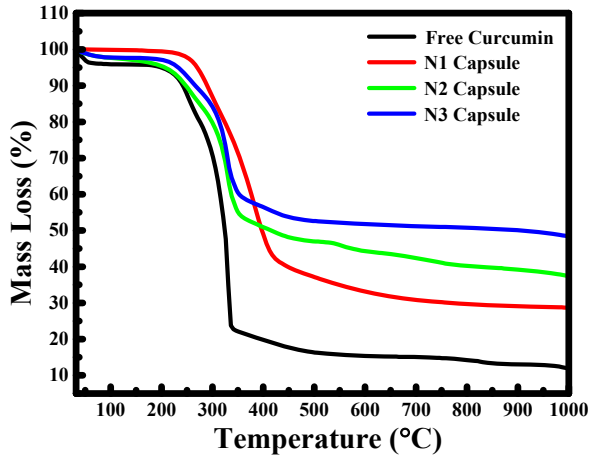

Figure 18: TGA spectra of DMPC lipid, N1, N2, N3 and Free Curcumin

The main difference was in the % mass loss of the 3 nanocapsules. It was found that the % mass loss for N1, N2 and N3 capsules are 64%, 54% and 40% respectively. This confirms the fact that curcumin is more encapsulated in N2 and N3 capsules than N1.

2.3.5. Drug delivery release

Various approaches have been developed in order to increase curcumin delivery and to protect it from degradation. Hence, different kind of therapeutic nanoparticles are designed to improve curcumin bioavailability toward target specific cells such as liposomes.[107] Since curcumin is hydrophobic, it can be easily encapsulated within the liposome bilayer. The release of curcumin out of the liposome bilayer depends on the modification of the nanocapsule surface and the pH of the medium. Initially, curcumin release was investigated by studying the effect of the additive layer (N1, N2 and N3), although the effect of the pH for each capsule was established to determine its effect on curcumin release.

2.3.5.1. Effect of additive layer

The release of the curcumin from the nanocapsules was recorded by measuring the absorbance in different interval time and adding the absorbance value consecutively. The release of curcumin for the three different cases N1, N2 and N3 at pH 7 is established in Figure 23. Hence, it is obvious that the release rate of the curcumin is directly affected by the addition of the coated layer.[108]

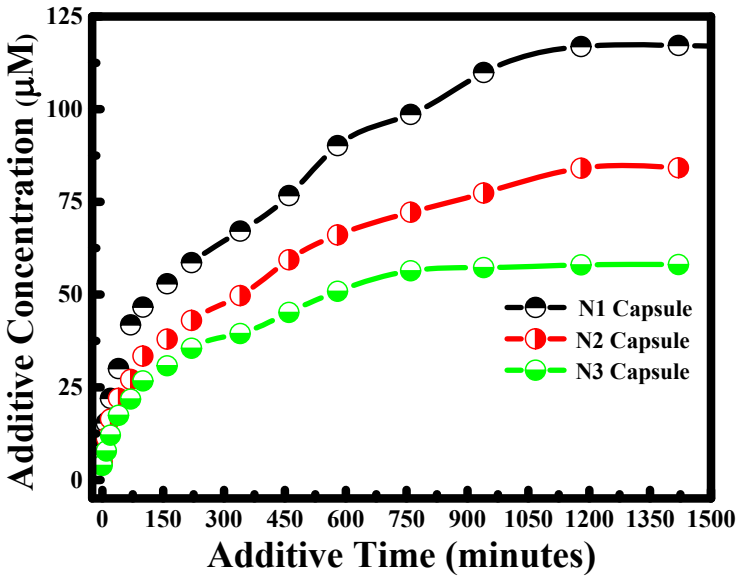

Figure 19: Effect of Additive layer on the release of curcumin in N1, N2 and N3 at pH7.

In this context, it was predicted that when the number of layers increases the curcumin release decreases. Thus, it is clear from the plot that, faster release of the drug is assumed when the liposome surface is only coated by PDDA layer. However, the rate of release decreases by adding at the first silica nanoparticles and decreases again by adding curcumin exhibiting the slower drug release. Furthermore, in all three cases the drug release increased during the drug delivery mechanism that was held for two consecutive days, where it reaches its maximum release for N1 and its minimum release for N3. Such layer by layer assembly enhances the stability of the nanocapsule due to the electrostatic interaction between the oppositely charged polymer and the surface of the nanocapsule.[109] Consequently, the stability of the nanocapsule increases when its surface is coated with more layers as our results explain.

When silica is incorporated between two polymeric layers, more electrostatic interactions are exhibited. Therefore, the negatively charged silica nanopartilces interacts with the two layers of the polymer forming stronger electrostatic interaction. Moving on to the third case, the addition of curcumin just after silica, delayed the release of curcumin encapsulated within the liposome. Hence, the multiple layers boost the diffusion distance thus hindering contact between the drug and the release media considerably for a longer time.[110] Moreover, It is clear that at t = 0 minutes the absorbance was equal to 0 for N2 and N3 indicating that there was no drug release. Thus, there was a noticeable curcumin release for N1 at the same time where the absorbance was equal to 0.8 revealing the existence of short length path.

2.3.5.2.Effect of pH

Different layers were added to the nanocapsules in order to get N1, N2 and N3. However, all the nanocapusules were at the end coated with a PDAA layer. In consequence, the modification of the pH will alter initially the final layer present which means the PDAA layer. Three different pH were tested accordingly (4, 6 and 7).

Nowadays, measuring the surface charge of the tested samples is considered the most flexible method. In fact, zeta-potential is an easy and simple technique to understand the phenomena that occur on the surface of the charged moieties.[111] Zeta potential is used to find the electrokinetic potential in colloidal systems, which has a considerable impact on the different characteristics of nanocarrier systems. Testing the surface charge is a dominant role which could extremely effect on the mode of release of for the encapsulated agent.[112]

To understand the mode of interaction between each layer in the self-assembly, Zeta-potential titration analysis was done for N1, N2 and N3 at different pH. For this purpose, after

centrifuging the nanocapsule, the precipitate was dissolve in pH equal to 3 and automatically NaOH (3 mM) solution was added to the solution to achieve the desired pH and the value of the charge surface was monitored directly. Zeta potential of curcumin alone was also investigated to evaluate the interaction between curcumin and PDAA layer.

The effect of three different pHs 4, 6, and 7 on the release of curcumin for N1, N2 and N3 is depicted in Figures 24 A, B&C. The drug release graphs show the change in the concentration of curcumin during 24 hours, where the concentrations were calculated based on the calibration curves of curcumin at pH 4, 6 and 7 using Lambert-Beer's law.

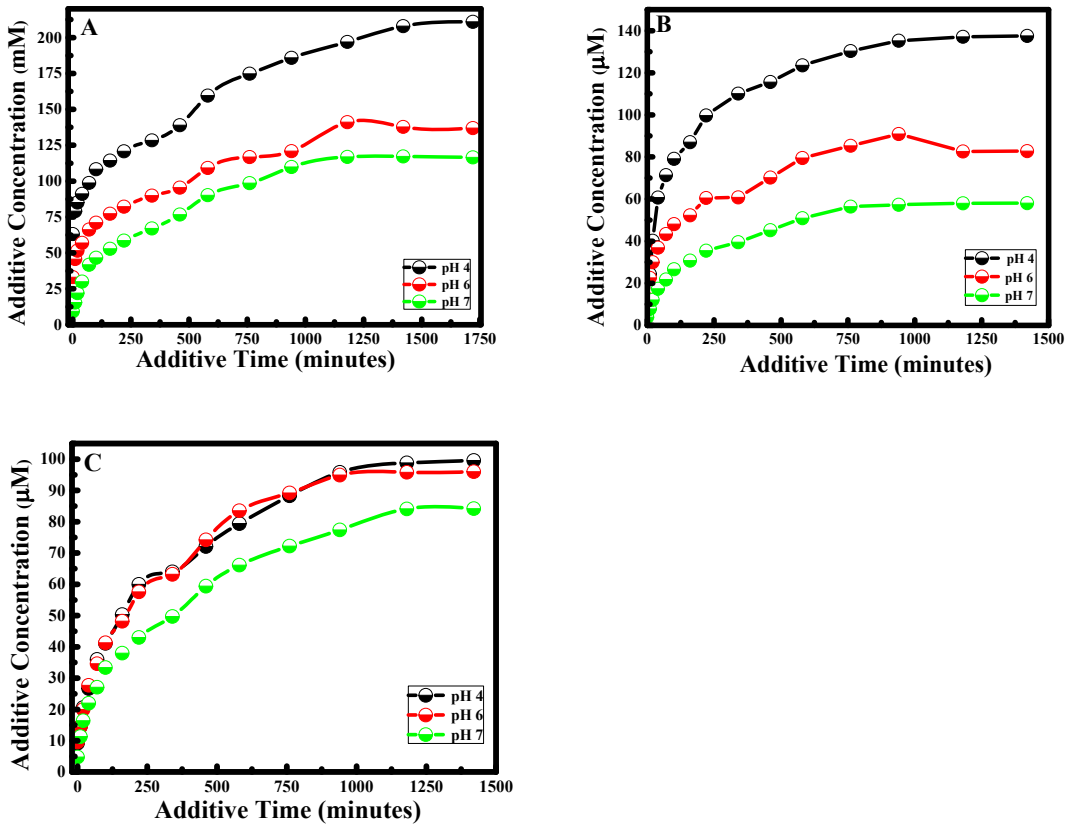

Figure 24: Drug release for (A) N1, (B) Drug release for N2, and (C) for N3 capsule at pH 4, 6 and 7.

Zeta potential measurements of curcumin show that the surface of curcumin is positively charged at pH=4, while it is negatively charged at pH equal to 6 and 7. For the three nanocapsules, the highest release was obtained for pH 4, medium release was observed at pH 6 and the lowest release at pH 7. It is shown that at high acidic conditions (pH=4), drug release results demonstrated high delivery of curcumin from the nanocarrier system. This refers to the same positive charged surface of both curcumin and the membrane surface of the nanocapsule at pH 4 inducing the inhibition of the curcumin entrapping in the membrane. Studies showed that tumor tissues contain acidic gradients different from the normal tissues, which consist of alkaline gradients.[113] In addition, it was demonstrated that the cellular surface of the malignant tissues are negatively charged.[114] Thus, in our case it is expected to have high cellular uptake of curcumin by the tumor cells due to the electrostatic interaction between the curcumin and the cell surface. Additionally, recent results have emphasized that the positively charged nanoparticles are preferably absorbed by the malignant tissues,[115] and stored for a longer period of time in comparison with particles of negative or neutral surfaces.[116]

However, under lower acidic medium for pH 6 and pH 7, curcumin demonstrated lower drug release. Zeta-potential measurements imply positively charged membrane surface of nanocapsule and negatively charged surface of curcumin at pH 6 and 7. Thus, the electrostatic interaction between the oppositely charged particles of the drug and nanocapsule enhanced the encapsulation efficiency of curcumin. Although, curcumin remained entrapped in the nanocapsule during the drug delivery process. Hence, the release of curcumin at pH 7 was lower than the release at pH 6. This difference in the drug release is due to the highest surface charge obtained at pH 7 for the three nanocapsule (Table 2).

Table 2: Zeta potential value for Free curcumin, N1, N2 and N3.

pH / Nanaocapsule	N1	N2	N3	Curcumin
4	+23.9	+42.8	+44.7	+2.3
6	+25.4	+24.5	+22.7	-2.3
7	+21.8	+30.3	+31.9	-4.1

In general, large positive value of zeta potential reflects good and high physical stability of nanocapsules due to electrostatic repulsion of individual particles. Hence, when zeta potential value is above +30 mV, it is generally considered to have sufficient repulsive force to attain better physical colloidal stability, enhancing the encapsulation of curcumin and thereby lower release of the drug. On the other hand, a small zeta potential value can result in particle aggregation and flocculation due to the Van der Waals attractive forces and thereby higher release of the drug.[117] Moreover, the drug release study at pH 4 and 6 did not have any effect on the nanocapsule itself, since lower release of curcumin was also obtained for N3 at pH 4 and 6, similar to the results obtained at pH 7 described in the previous Section.

2.3.6. Cytotoxicity study by MTT proliferation Assay

Recent study has calculated the IC50 of curcumin to be 25±5.2 μM.[118] This concentration was sufficient to kill 50% of the cancer cell after 48 hours. To establish the effect of the different nanocapsule on the breast cancer cell, 25 μM of N1, N2, N3, curcumin, silica nanoparticles, PDAA and DMPC liposomes were used to treat the cancerous cell within 72 hours. To measurement of the cytotoxicity of the above reagents on breast cancer cells, MTT proliferation

assay was used. As shown in Figure 25, N3 exhibits the high % of inhibition. Hence, 90% of the cells were killed within 72 hours when treated with N3. However, N1 and N2 reveal 73 and 87 % of inhibition.

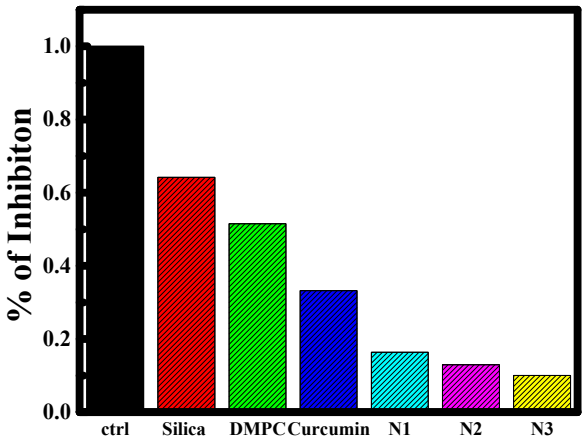

Figure 25: Effect of Silica, DMPC liposome, Curcumin, N1, N2 and N3 on the inhibition % of MCF-7 cancel cells.

The difference in the effect of the three nanocapsule regarding in the % of inhibition, refers to the curcumin percentage encapsulated in the core of liposomes. However, N3 exhibits the high encapsulation efficiency and thereby the highest percentage of inhibition. Although, curcumin alone killed 57% of the cancer cells. Hence, the nanocapsule enhances the effect of curcumin as anticancer reagent.

2.4. Conclusion

Our target in this chapter was to boost the curative activity of curcumin by encapsulating it in a DMPC liposome based nanocapsule using self-assembly method. The characterization of the nanocapsules including SEM, DLS, TGA, XRD and spectroscopic techniques have shown a

good agreement. Hence, it was proved that curcumin established more encapsulation in the nanocapsule with higher electrostatic interaction. In this direction, it was confirmed that curcumin is strongly entrapped in the lipid by layer, as the surface of liposome is coated with multi layers. Moreover, the effect of the added PDDA was best understood by analyzing the interaction between PDDA and the encapsulated curcumin through XRD. Thus, XRD patterns manifested the variation in the packing structure of curcumin. The cytotoxicity of the nanocapsules, curcumin, silica and PDDA was performed using MCF-7 malignant cells. Results, demonstrated that N3 capsule exhibits the highest % of inhibition, where 90% of the cells were killed within 72 hours. However, lower percentages were revealed for N2 and N1. Thereby, this refers to the high amount of curcumin accumulated in the core of N3 capsule, which is attributed to the high encapsulation percentage.

CHAPTER 3

DMPC AND DPPC MIXTURE LIPOSOMES BASED NANOCAPSULES FOR CURCUMIN DELIVERY

3.1. Introduction

Studies show that the formation of typical liposomes with preferable stability will boost drug delivery. Hence, adequate dose of the encapsulated drug will be liberated to the target cells or organs. The stability of the liposome is best linked with the amount of the drug released from the system which is directly proportional to the length of hydrocarbon chain of the used lipid. In this direction, it was proved that the phase transition temperature of the liposome as well as the length of its acyl chain influence greatly on the stability of the liposomal drug delivery system.[119] In this CHAPTER, we will introduce a combination of two saturated liposomes DMPC and DPPC, with three different ratios. The prepared ratios are as follows, 50:50 (DMPC : DPPC), 75:25 (DMPC : DPPC) and 25:75 (DMPC : DPPC) mixtures. The all formed liposomal curcumin were coated with PDDA later. The nanocapsules were analyzed using DLS, SEM, Zeta-potential and Spectroscopic techniques. Later on, the drug release profile was analyzed during 24 hours for each nanocapsule at three different pHs (4, 6 and 7).

3.2. Materials and Methods

3.2.1. Materials

Curcumin, HS-40 Colloidal Silica, polydiallyldimethyl ammonium chloride (PDAA) and ethanol were obtained from Sigma-Aldrich. The phospholipids including 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine were

obtained from Avanti. Chloroform was purchased from Sharlu. Buffer solutions with a pH equal 4, 6 and 7 were purchased from Fisher. The chemicals were used directly without any purification. DPPC, DMPC and curcumin were dissolved in ethanol and chloroform and HS-40 Colloidal Silica was dissolved in double distilled water.

3.2.2. Synthesis of liposomal curcumin

Liposomal curcumin were prepared using different DMPC:DPPC ratio. Three ratio of DMPC : DPPC were studied accordingly; 50:50, 75:25 and 25:75. Each ratio was prepared at 3 different pHs (4, 6 and 7). To start with, 1.84 mg of curcumin was dissolved in chloroform and ethanol with 1:1 ratio and added to the different phospholipids mixture, initially dissolved in 1:1 ratio of chloroform and ethanol. Using rotary evaporator at 60 °C, the organic solvents were evaporated until obtaining a thin film on the wall of the vial. Afterwards, the sample was kept in vacuum oven for 1 hour at 40 °C to eliminate all the traces of the organic solvents. Later on, 5 mL of buffer solution was added in order to dissolve the film. The solution was vigorously vortexed and heated for 10 minutes at 51 °C (10 °C above the phase transition temperature of DPPC). To reduce the size of the liposomes, Mini-Extruder device from Avanti was applied for 20 times. 3 ml of 1 mg/mL of PDDA were added to 3 mL of the final solution and kept in water bath for 30 minutes at 51 °C followed by 30 minutes at room temperature. After that, the sample was centrifuged and 5 mL of buffer solution was added to the precipitate to get the nanocapsule.

3.2.3. Characterization technique and Spectroscopic measurement

The characterization techniques are as given in CHAPTER II.

3.2.4. Drug Delivery Release

After preparing the nanocapsules, the release of curcumin was studied at 37°C according to the human body temperature. The release of curcumin was studied for consecutive 48 hours. The nanocapsules were kept at 37°C for 1 hour and then centrifuged at 15000 rpm for 15 minutes. The absorbance of the supernatant was measured and 5 mL of buffer solution were added to the supernatant and kept for 1 hour at 37 °C and then centrifuged again. This step was done for several times and the absorbance of the supernatant was always measured.

3.2. Results and Discussion

3.2.1. Preparation of the nanocapsule

The two phospholipids and the curcumin were dissolved in ethanol and chloroform with a ratio to 1:1. The evaporation of the solvent was done at 60 °C until a uniform film appears. 5 mL of buffer solution was added to dissolve the film and thereby the formation of the liposomal curcumin. After addition of buffer solution (pH 4, 6 or 7), a positively charged polymer layer was added on the liposome surface in a way to increase the stability of the liposomal curcumin.

3.2.2. Characterization and Spectroscopic Measurements

The shape of the three mixture ratios; 50:50; 75:25 and 25:75 of DMPC : DPPC were analyzed using SEM. When equal concentration of DMPC and DPPC were used uniform and identical spheres were obtained with size range between 80-130 nm (See Figure 26A). However, when varying the concentration of the phospholipids in a ratio of 75:25 DMPC: DPPC, dispersed spheres were obtained with smaller size <85 nm (Figure 26B). Although, for 25:75 DMPC: DPPC ratio (Figure 26C), bigger spheres were obtained with diameter higher than 100 nm.

Hence, DPPC liposome is resulting in the formation of larger spheres, where DMPC liposome conducts to the presence of smaller nanocapsules.

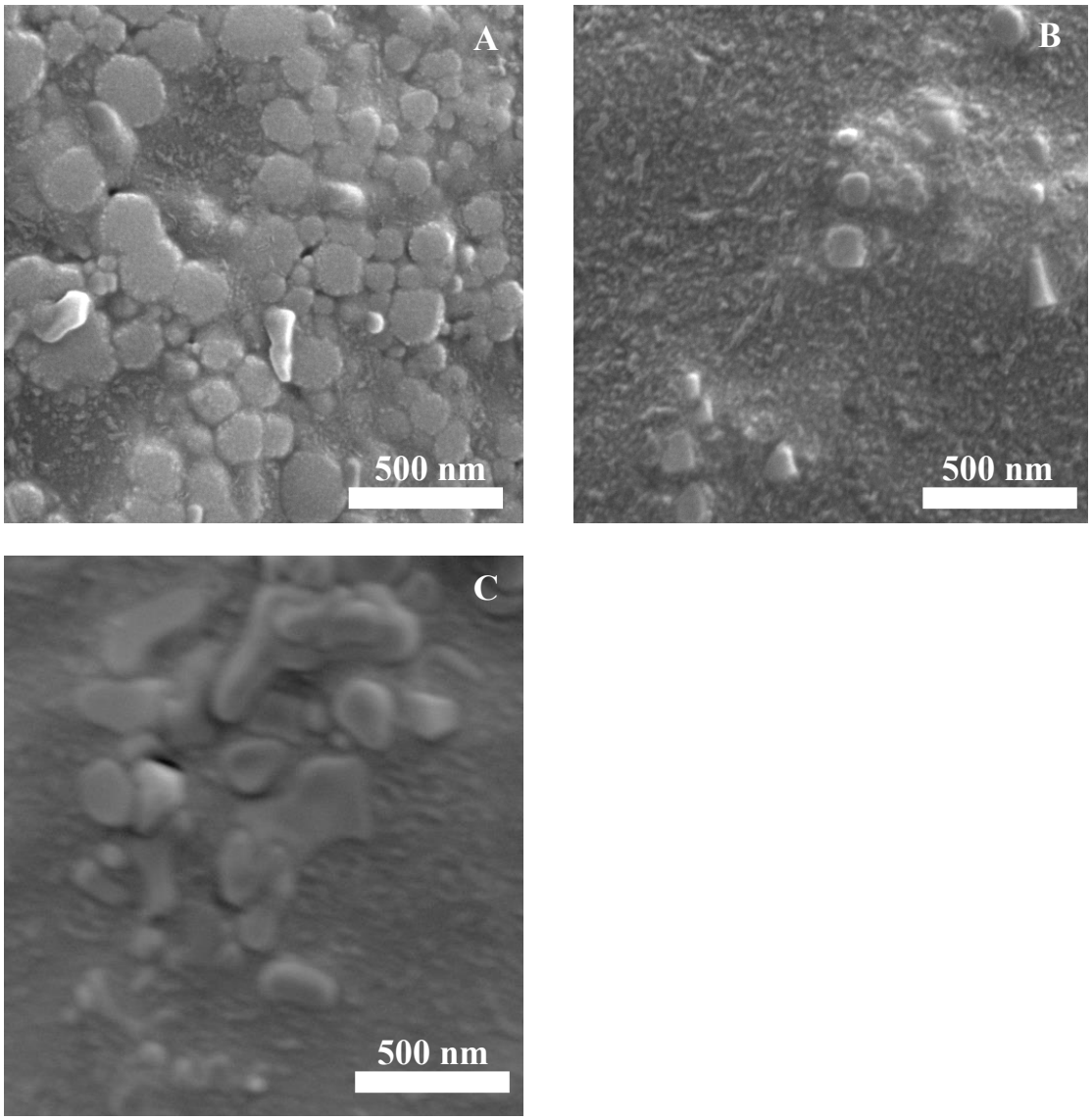

Figure 20: SEM images for (A) 50:50, (B) 75:25 and (C) 25:75 ratio of DMPC:DPPC.

Dynamic light scattering analysis (DLS) gave the same trend of the size for the 3 different mixtures with polydispersity equal to 0.320. For 50:50 ratio the particle diameter size was 725.3 nm, for 75:25 ratio the size was equal to 424.6 nm and finally for 25:75 nm the

particle diameter size was equal to 507.4 nm. Hence, the difference in the size between DLS and SEM is due to the fact that DLS measures the size of several particles.

The absorbance of the three nanocapules after centrifuging and curcumin was measured and the results are depicted in Figure 27.

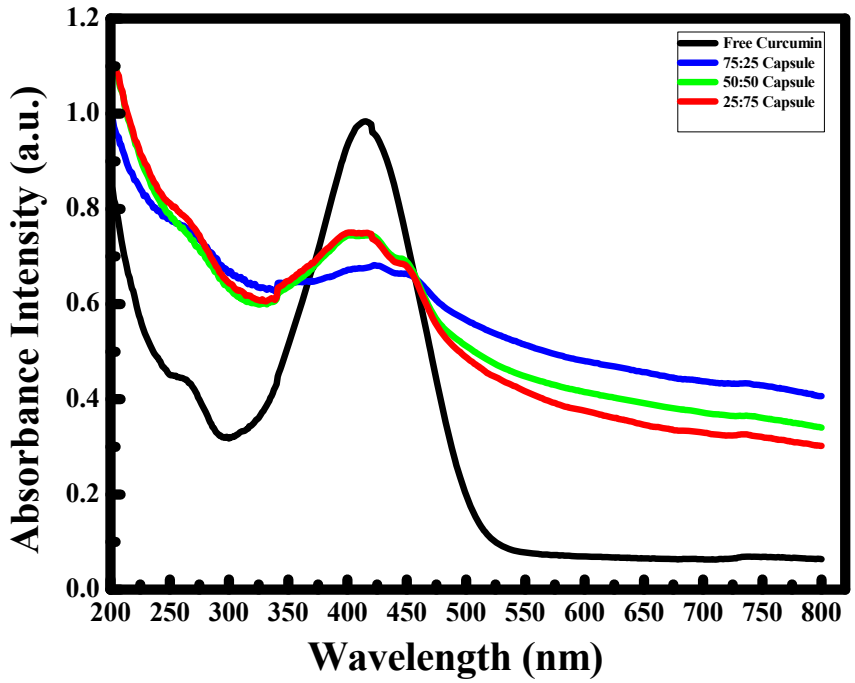

Figure 21: UV-Visible spectra for Free curcumin, 75:25, 50:50 and 25:75 ratios of DMPC:DPPC.

The characteristic peak of curcumin was found to be at λ=425 nm for free curcumin as for the three different ratios. However, the main difference was in the shape of the peak, where the sharp peak of free curcumin became broader while adding polymer to the surface of liposomal curcumin. In addition, the absorbance of the three different ratios was less than the absorbance of free curcumin. In fact, 50:50 and 25:75 ratios of DMPC: DPPC exhibit same absorbance intensity which was equal to

0.78 a.u, higher than the absorbance value of 75:25 ratio equal to 0.69 a.u. Thereby, in 25:75 ratio and 50:50 ratio of DMPC: DPPC, curcumin is being more encapsulated than 75:25 ratio. This difference is due to the type of liposome which is dominated. Hence, in 25:75 ratio DPPC liposome exhibits 75% of the total added liposome which is composed of 40 carbon atoms, while DMPC contain 36 carbon atoms. Hence, the longer carbon chain present in DPPC induces the entrapment of curcumin into the liposomes bilayers and therefor enhances the encapsulation of curcumin. Moreover, the fluorescence emission intensity was measured for free curcumin and for the three different ratios at two definite excitation wavelengths λ=425 nm and λ=350 nm. The results are shown in Figures 28A&B.

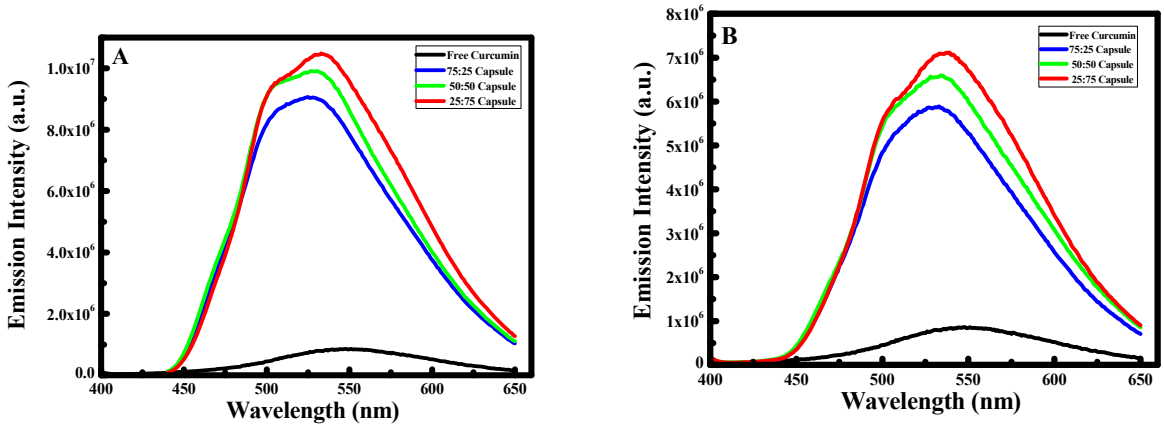

Figure 22: (A) Flurescence emission spectrum excited at λ = 425 nm, (B) Flurescence emission spectrum excited at λ = 350 nm for free curcumin, 75:25, 50:50 and 25:75 ratios of **DMPC: DPPC.**

In fact, curcumin exhibits a definite peak at λ=550 nm of both excitation wavelengths. A blue shift to λ=527 nm occurs in the three different ratios. This blue shift is as a result of the addition of polymer layer verifying the encapsulation of curcumin in the liposomes. The 25:75 ratio of DMPC: DPPC, demonstrates the higher emission intensity compared to 50:50 and 75:25

ratio DMPC: DPPC mixture. These results confirm the difference in the absorbance obtained for the 3 different ratios. However, the emission intensity emphasizes the effect of the liposomes concentration where, when more DPPC is added, more curcumin is encapsulated. The difference in the 50:50 ande 25:75 ratio was remarkable in the emission intensity spectra verifying the fact that when higher concentration of DPPC is added to the mixture the curcumin is being more encapsulated, regarding the presence of longer carbon chain. Resonance Rayleigh Scattering (RRS) was also measured and the results are depicted in Figure 29.

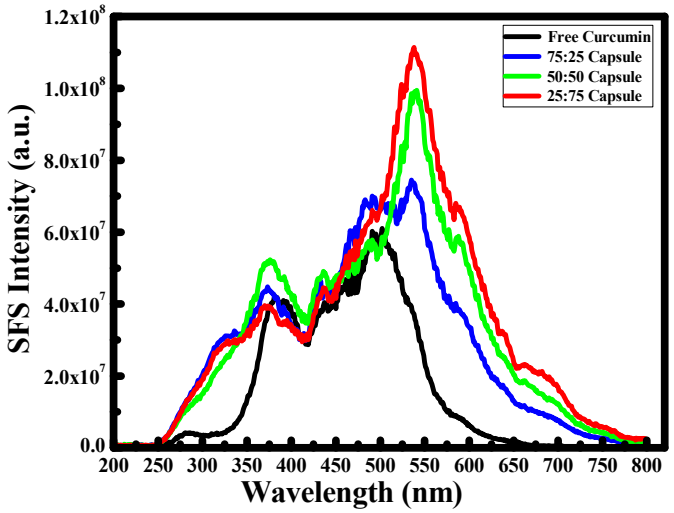

Figure 23: Synchronous fluorescence spectrum for free curcumin, 75:25, 50:50 and 25:75 ratios.

Free curcumin spectra presents two significant peaks at λ = 385 nm, which is shifted to λ = 375 nm and a strong peak at λ = 500 nm shifted to λ = 535 nm. Hence, the blue and the red shift obtained in the RRS intensity, reflects the presence of the encapsulation of the curcumin in the core of liposomes. In addition, the highest intensity was obtained for the 25:75 ratio of DMPC:DPPC, confirming the maximum encapsulation effeciency obtained in this case.

3.2.3. Effect of pH on the Drug Release Activity

The drug delivery profiles were carried out at T = 37 ℃ and analyzed using Uv-Visible measurement after centrifuging at 15, 000 rpm for 15 minutes for 24 hours. Figures 26 A, B&C illustrate the effect of three different pHs 4, 6, and 7 on the release of curcumin from 50:50 ratio, 75:25 ratio and 25:75 ratio capsules. The effect of curcumin's release at pH = 10 was done but not included in this section, since no biological application was explained at alkaline conditions.

As it is shown in Figure 30A for 75:25 ratio mixtures of DMPC:DPPC, it is obvious that under acidic medium, curcumin manifests the higher release (260 μM), in comparison with pH 6 and pH 7. Based on zeta-potential measurements, results illustrated positively charged surface of capsule at pH 4 in consistent with the curcumin's surface charge. However, the liberation of curcumin drug is lower at pH 6 and at pH 7, where it is nearly 180 μM and 150 μM, respectively. The decrease in the curcumin's delivery is related to the positively charged surface of the nanocapsule, which is opposite to the charge of curcumin, which induces stronger interaction and in consequence lower release. Moreover, Figure 30B&C depict curcumin's delivery for 50:50 and 25:75 ratio mixtures, respectively.

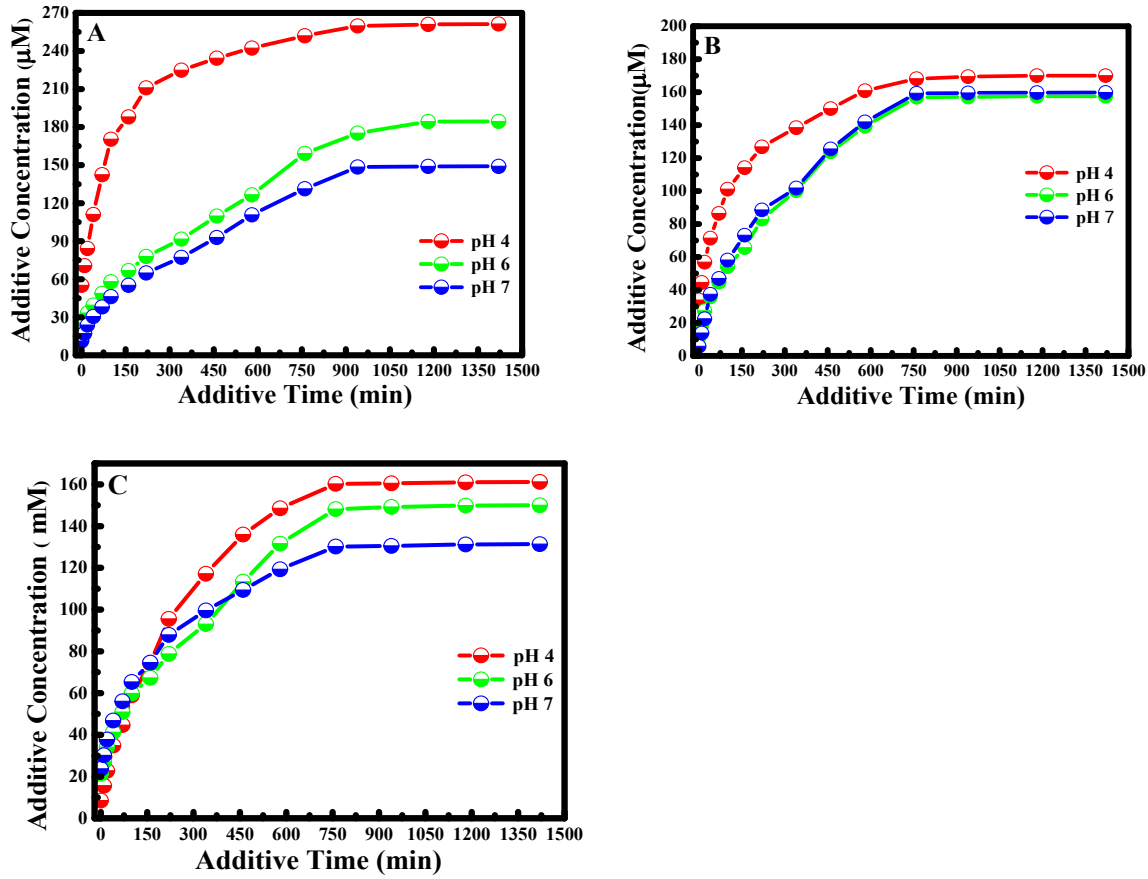

Figure 30: Drug release for (A) 75:25, (B) for 50:50 and (C) for 25:75 ratios of DMPC:DPPC. at pH 4, 6 and 7.

The release of curcumin in both capsules, show the same delivery profile as 75:25 mixture during 24 hrs. In 50:50 ratio, the final concentration of released curcumin was 170 µM for pH 4 and 160 µM for both pH 6 and pH 7. Whereas, the drug release graph in 25:75 capsule exhibits the lowest concentration in comparison with the other mixture capsules. The final concentration that was reached at pH 4, pH 6 and pH 7 is nearly 160 µM, 140 µM and 120 µM; respectively. Thus, less curcumin was released from the capsule containing higher concentration of DPPC liposome. The difference in the drug release depends strongly on the difference between the charge of curcumin and the nanocapsule. Hence, at pH equal to 4, curcumin and the

nanocapsule exhibit positive charge. The same surface charge inhibits the entrapping of curcumin into the core of the liposomes. However, when the surface charge of curcumin is negative, opposite to the charge of the nanocapsule surface, curcumin is forced into the core of the liposomes and thereby the drug release decreased. In this case, the highest positive value results in stronger interaction and thereby lower release. The difference in the zeta values are summarized in Table 3.

Table 3: Zeta potential value for Free curcumin, 75:25, 50:50 and 25:75 ratios of DMPC: DPPC.

pH / Ratio	72:25	50:50	25:75	Curcumin
4	+19.3	+34.8	+36.5	+2.3
6	+20.1	+23.1	+23.6	-2.3
7	+11.6	+13.1	+17.1	-4.1

3.3. Conclusion

Our target in this chapter was to manifest the effect of using a combination of two types of lipid while monitoring curcumin's release from the nanocapsules. The shapes of the mixture capsules were conducted by SEM, where uniform and well-shaped spheres were appeared in 50:50 DMPC: DPPC ratio. However, dispersed and small size spheres <85 nm were presented for 75:25 DMPC: DPPC ratio and bigger spheres were obtained for 25:75 DMPC: DPPC with diameter higher than 100 nm. This variation is linked to the variation of tail group between DPPC and DMPC lipids. Spectroscopic measurements have shown that 25:75 mixture revealed

the lowest intensities among the tested nanocapsules. Thus, curcumin is best encapsulated in this ratio, this refers to the fact that the long chain of DPPC liposome could boost the encapsulation efficiency of curcumin. Meanwhile, DPPC liposomes have shorter acyl chain (C_{14}) than DPPC liposomes (C_{16}).

CHAPTER 4

LIPOSOMES BASED NANOCAPSULES FOR SELECTIVE SENSING OF ATP

4.1. Introduction

The application of nanoparticles as detectors for biomolecules using fluorophore has enhanced the specificity and selectivity.[38] Besides the therapeutic activity of curcumin as anti-oxidant, anti-inflammatory and anti-caners agent, curcumin was greatly used as a fulorescent probe in optical sensing targeting several analytes.[79,81,120] In this work, we will assess the ability of curcumin entrapped in DMPC liposome nanocapsule to detect ATP molecule. The properties of DMPC capsule was conducted using spectroscopic techniques, DLS, Zeta-potential, SEM, TGA and XRD. The detection method was carried out by measuring the emission intensity of each sample with different ATP concentration ranges between 0 μM and 100 μM. Furthermore, selectivity study will be presented, by following the same method on analogous biomolecules such as, GTTP, TTP, CTP and UTP.

4.2. Materials and Methods

4.2.1. Materials

Curcumin, HS-40 Colloidal Silica, poly (diallyldimethyl ammonium chloride) and ethanol were obtained from Sigma-Aldrich. 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was secured from Avanti. Chloroform was obtained from Sharlu. Buffer solutions with pH equal to 7 was purchased from Fisher. Adenosine Triphosphate (ATP) was obtained from Sigma. All chemicals were used directly without further purification and were dissolved in double distilled water.

Curcumin and DMPC, were previously dissolved in ethanol and chloroform since they are insoluble in water.

4.2.2. Synthesis of Liposomal Curcumin

The synthesis of liposomal curcumin was performed as described before following thin film hydration procedure. In the first step, 1.84 mg of curcumin was initially dissolved in 0.5 mL of ethanol and 0.5 mL of chloroform. Later on, curcumin solution was added to 10 mg of DMPC which was dissolved in 2.5 mL of ethanol and 2.5 mL of chloroform. In a second step, the organic solvents were evaporated using rotary evaporator at 60 ℃, where thin film formation was observed after complete evaporation. Followed by keeping the sample in vacuum oven for 1 hour at 40 ℃, in order to eliminate the traces of the organic solvents. Afterwards, 5 mL of buffer solution of pH 7 was added and vigorously vortexed and heated for 10 minutes at 34 ℃, until obtaining a complete homogeneous solution. In order to get small size of liposomal curcumin particles, Mini-Extruder device from Avanti was applied for 20 times.

4.2.3. Preparation of Nanocapsule

The nanocapsule, which was used for sensing of ATP, was prepared as described below. All the centrifuging runs were done at 15, 000 rpm for 10 minutes. In the first step, 3 mL of liposomal curcumin was mixed with 3 mL of PDDA. Then, the nanocapsule was kept for 30 minutes at 34℃ followed by another 30 minutes at room temperature and then centrifuged. In the second step, 30 µl of silica nanoparticles were inserted and the sample was set at room temperature for 1 hour and centrifuged. In the third step, 3 mL of buffer solution and 3 mL of 1 mg/mL of PDDA solution were added and mixed properly using sonication. The nanocapsule

then was kept for 30 minutes at 34 ℃ and 30 minutes at room temperature and then centrifuged. Later on, an additional layer of silica was added and the solution was kept undisturbed for 1 hour and centrifuged. Finally, the nanocapsule was coated with a PDAA layer. The preparation was done at neutral pH using buffer phosphate solution.

4.2.4. Characterization and Spectroscopic Measurements

The characterization techniques are as given in CHAPTER II.

4.2.5. ATP detection by DMPC nanocapsule

DMPC nanocapsule was dissolved in 10 mL of PBS solution. A volume equals to 0.2 mL of the prepared stock solution was taken for the detection of ATP. A stock of 150 μM of ATP solution was prepared in 15 mL DDW. Each time a definite volume was taken from ATP stock solution and was added to 0.2 mL of DMPC nanocapsule to prepare different concentrations of ATP ranging between 0 μM and 100 μM. DDW was added each time to have a 3mL solution as a final volume. Similarly, 150 μM stock solutions of GTP, UTP, CTP and TTP analytes were prepared for interference study.

4.3. Results and Discussion

4.3.1. Nanocapsule preparation

As obtained in Chapter II, the N3 nanocapsule prepared with 2 layers of PDAA polymer, one layer of curcumin and one layer of silica nanoparticles, resulted in the highest encapsulation efficiency and lower drug release. For sensing purpose, it is better to use nanocapsule which has less drug release. Hence, the nanocapsule prepared for ATP detection was composed of 3 PDAA

layers and 2 silica nanoparticles layer to assure more encapsulation of curcumin into the liposome core, (figure 31).

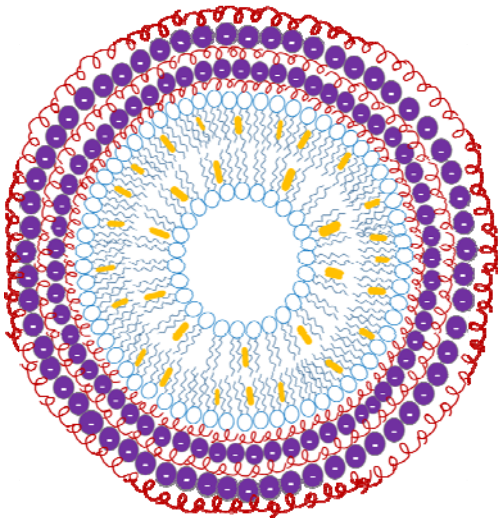

Figure 31: Schematic Illustration of DMPC Nanocapsule.

4.3.2. Characterization and Spectroscopic Measurements

Surface morphology of sensing nanoparticle was carried out using SEM as depicted in Figure 32. Results illustrate that the obtained nanoparticles have spherical shapes with diameter varying between 90 nm and 150 nm, with a uniform distribution.

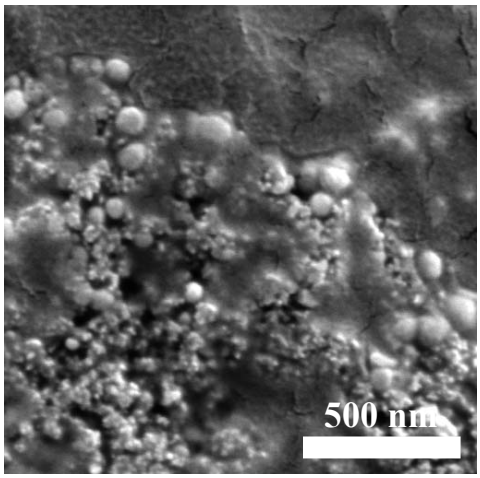

Figure 24: SEM image for the prepared nanocapsule.

Moreover, the particle size was analyzed by DLS, where the prepared sample was diluted and sonicated before measuring. The obtained particles size is equal to 493.6 nm with polydispersity index of 0.296. The difference in the size of the nanoparticles between DLS and SEM results is due to the aggregation of the nanocapsules that occurred in the solution.

In addition, the surface charge of the studied DMPC nanocapsule was determined using zeta-potential. Measurements manifest a positively charged surface (5.92 mV) for the prepared nanocapsule (Figure 33). This is related to the positive polymer PDAA which was used as a final coating layer for the surface. Although, silica nanoparticles are positively charged, which make the curcumin surrounded by positively charged layers, inhabiting its release from the core of the liposome.

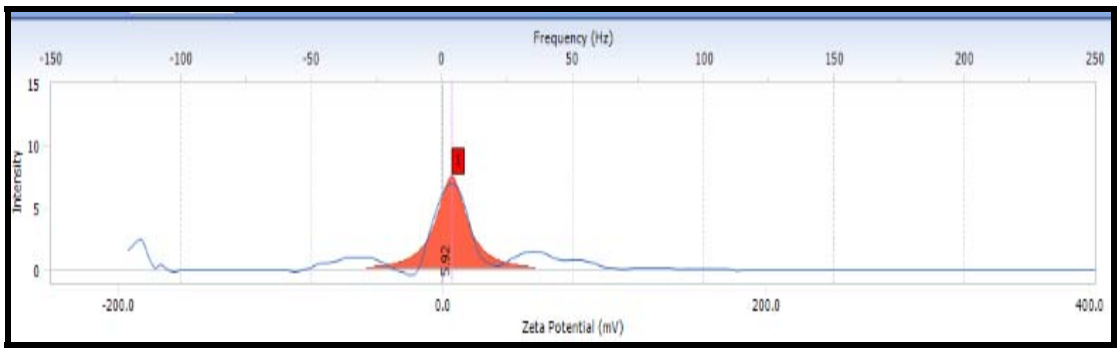

Figure 33: Zeta potential analysis for the prepared nanocapsule.

DMPC sensing nanocapsules were analyzed using UV-visible spectra, as shown in Figure 34A. Free curcumin exhibits a definite and narrow peak with a maximum absorbance ~ 1 (a.u.) at λ= 425 nm. DMPC nanocapsule shows a broad peak at λ = 425 nm with 0.7 (a.u.) absorbance intensity. The occurrence of a broader peak in the sensing nanoparticle, indicates that the curcumin is being highly encapsulated within DMPC liposome bilayers, which is tightly coated with several layers of PDDA and silica nanoparticles. SFS spectra illustrates a characteristic peak for free curcumin at

λ=385 nm which is blue shifted to λ= 375 nm, another significant peaks appeared at λ= 500 nm which is red shifted to λ= 550 nm. An additional two peaks occurred in DMPC nanocapsule at λ= 430 nm and at λ= 475nm. The blue and red shift as well the appearance of new peaks in DMPC nanocapsule demonstrated the fact that curcumin is tightly encapsulated in the core of liposome. (Figure 34B).

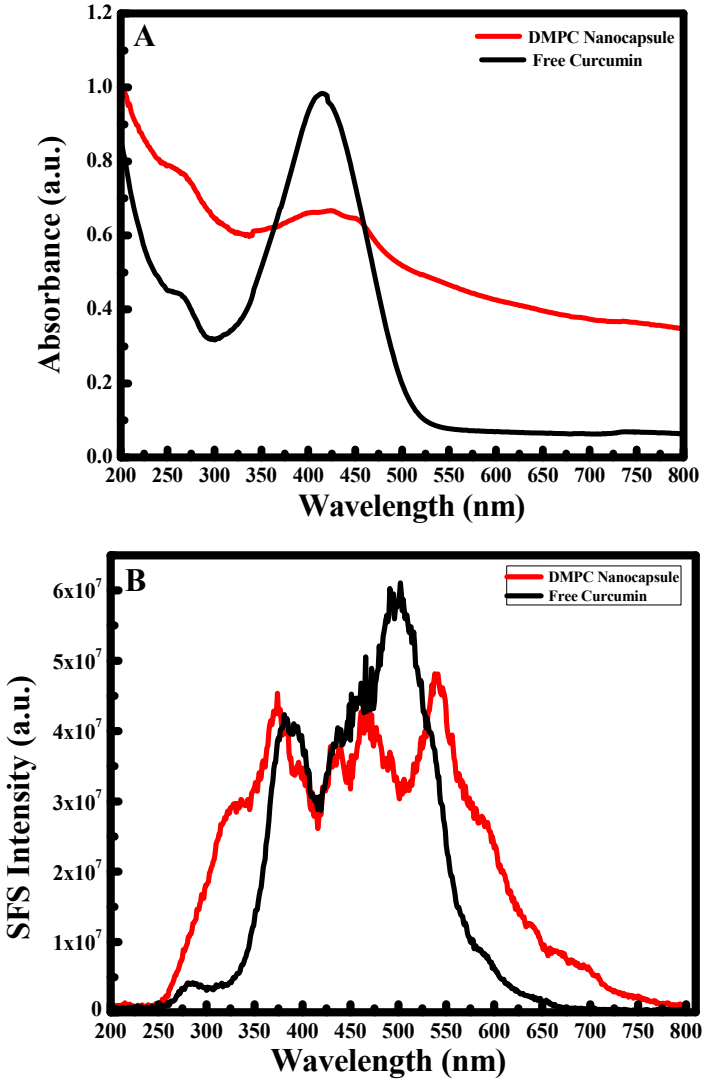

Figure 34: (A) UV-Visible and (B) SFS spectra of free curcumin and DMPC nanocapsule.

The X-ray diffraction was performed to examine the crystallinity of curcumin encapsulated in the nanocapsule. Figure 35 presents the XRD patterns of free curcumin, free DMPC lipid and the curcumin-DMPC nanocapsule.

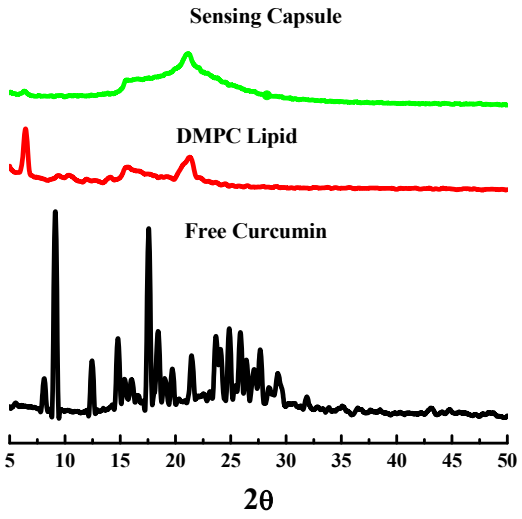

Figure 25: XRD pattern of free curcumin, DMPC lipid and DMPC nanocapsule.

The diffractograms of free DMPC lipid display the appearance of three characteristics XRD peaks at 2Θ equals to 6.4°, 15.7°, and 21.3°. Curcumin XRD patterns manifested significant peaks at diffraction angle equal to 8.06°, 9.20°, 12.46°, 14.95° and 17.75°.[104] It is found that the characteristic peaks of curcumin are almost absent in the XRD patterns of the nanocapsule. In addition, the DPMC peak present at 6.4° is absent in the diffractogram of the nanocapsule. The two peaks for DMPC present at 15.7° and 21.3° show one broad peak appeared at 2Θ equal to 23.7° for the naocapsule. This suggests that, all the curcumin present is being entrapped in the core of the liposomes resulting in high encapsulation.

Moreover, (TGA) was performed to investigate the thermal properties and composition for both pure curcumin and the nanocapsule as presented in Figure 36.

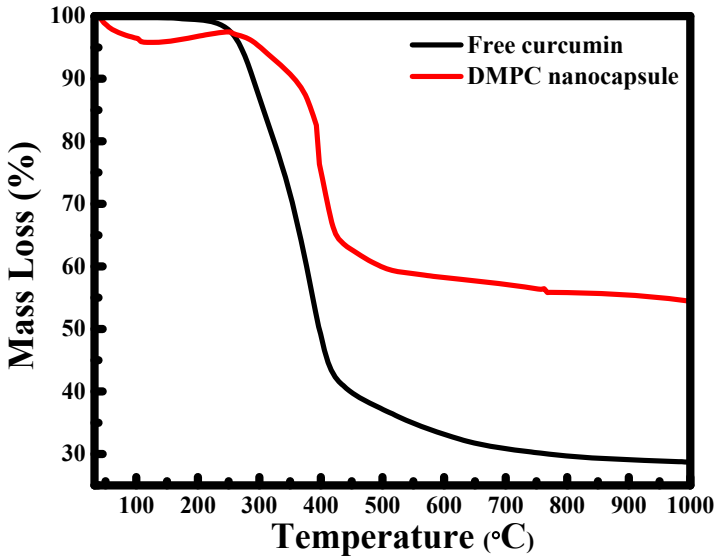

Figure 26: TGA analysis for free curcumin and DMPC nanocapsule.

The TGA pattern of pure curcumin shows that it undergoes one step thermal decomposition, with the first range 240- 560 ˚C.[99] However, it was shown that the nanocapsule illustrates mass loss between 30 ℃ and 100 ℃ attributed to the water loss, resulted from their hydroscopic feature. In addition, the nanocapsule exhibits degradation between 240 -560 ˚C. This loss is attributed to curcumin molecule. However, the % of curcumin mass loss in the nanocapsule is small than the % mass loss of pure curcumin. This is due to the fact that curcumin is being encapsulated in the core of the liposome, which induces its stability and protects it from degradation.

4.3.2. ATP detection by DMPC nanocapsule sample

The encapsulated curcumin in the prepared nanocapsule, was applied for ATP detection. It is obviously observed that the addition of ATP analyte to the nanocapsule solution, enhances the emission intensity. Thereby, the emission intensity of the nanocapsule increased by about five folds by

the addition of 5µM of ATP, where it remains increasing as the concentration of ATP increases. A blue shift is noticed from λ= 517 nm to λ= 500 nm as displayed in Figure 37A. Though, this shift could be related to the high surface free energy nanocapsules, where the ATP may be got adsorbed to the surface of nanocapsule. The rise in the emission intensity could be postulated to the increase in the aggregation of curcumin, as the concentration of ATP increase in the sample. Hence, ATP molecule binds through specific sites with the polymer molecule. The linear correlation of I/I_0 emission intensity of the DMPC nanocapsule as the function of ATP concentration (µM) is depicted in Figure 37B. The linear fit is in the range of 0-100 µM with a limit of detection equals to 0.11 µM. The increasing linear fit shows a linear equation, y= 0.1235 x + 3.74116 with $R^2 = 0.91158$ in the concentration range of 0-100 µM.

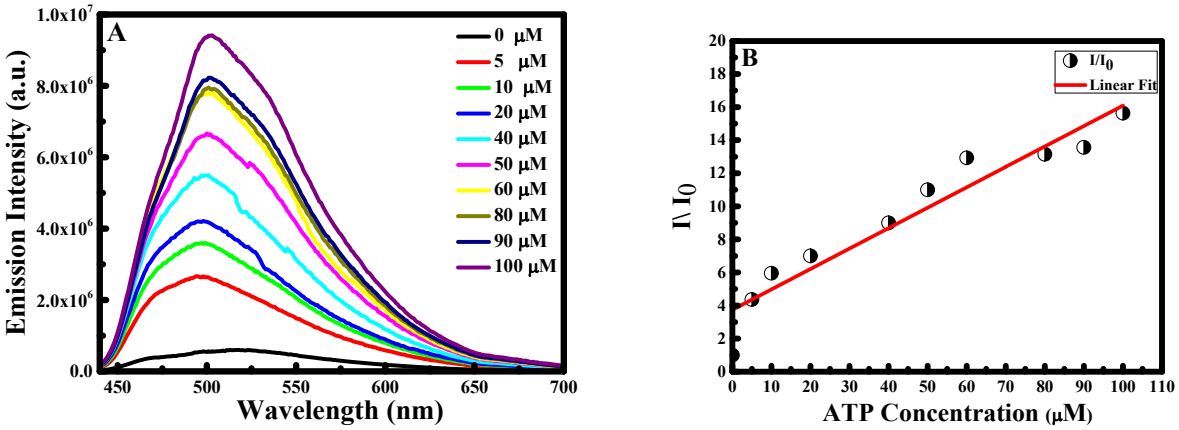

Figure 27: (A) Emission Spectrum excited at 425 nm for DMPC nanocapsule with different ATP concentration and (B) Linear correlation of emission intensity versus ATP concentration.

As the detection of ATP was possible by the nanocapsule, it is of a great importance to understand the mode of interaction between ATP molecule and the nanocapsule. In fact, Adenosine triphosphate, a small molecule, consists of a Five-Carbon sugar group which is located at the center, attached to a nitrogen adenine base group and to the chain of the three phosphate group.[121] As Zeta-

potential analysis revealed that the charge surface of nanocapsule is positive due to the existence of PDDA polymer in the surface. Thus, electrostatic interaction between phosphate group and quaternary ammonium group of PDDA could be attributed to the ability of nanocapsule to detect ATP.

In general, detection of ATP molecule is of a persistent clinical significance, though many studies and methods were done to detect ATP (Table 4). However, fluorescence detection is not widely used. Although, the detection of ATP is commonly done using analytical, calorimetric and electrochemical technique. Hence, these methods are expensive and requires the usage of several analytes.

Table 4: List of different method used to detect ATP.

Methods	LOD	Concnetration Range	Selectivity Study
Split Aptamer-Based Thioflavin T (ThT) replacement assay[122]	2 nM	0.1 μM - 120 μM.	GTP, UTP, ADP, AMP.
Gold nanoparticle based picogreen flurescent DNa Calculator[123]	46.5 nM	50 nM – 500 nM	GTP, UTP, CTP, ADP, AMP.
Surface energy transfer and flurscence recovery by rhodamine B and curcubit[7]uril-cappedgold nanoparticles assay[124]	100 nM	0.5 –10 μM	UTP, GTP, CTP, TTP, ascorbic acid, glutathione.
Mito-Rh flurescence probe[125]	0.033mM.	0 mM - 10.0 mM	AMP,GTP, CTP, UTP, inorganic anions .
DNA probes –Based Silica Nanoparticles[126]	34 μM	0 mM - 7 mM.	ATP, CTP, GTP, UTP.
ATP aptamer and single strandedDNA (ssDNA)-linked mesoporous silica nanoparticles (Si-MPs) complex, using AuNPs[127]	60 nM	60 nM - 1μM	ADP, AMP.
Colorimetric detection by ATP binding aptemers with unmodified gold nanoparticles[128]	50 nM	50 nM –1000 nM	UTP, CTP, GTP, NTP.
DMPC Mediated poly (diallyldimethylammonium chloride) Based Nanoparticles **(our Work)**	0.11 μM	0 μM -100 μM	UTP, GTP, CTP, TTP

The selectivity of the nanocapsule sensing was verified by measuring the emission intensity of the nanocapsule in the presence of biomolecules with similar structures to that of ATP like, UTP, GTP, CTP and TTP molecules (Figure 38). The results did not show an enhancement in the emission intensity of the nanocapsule, as it was shown in the case of ATP. Thus, the tested method is specific only for the detection of ATP biomolecule.

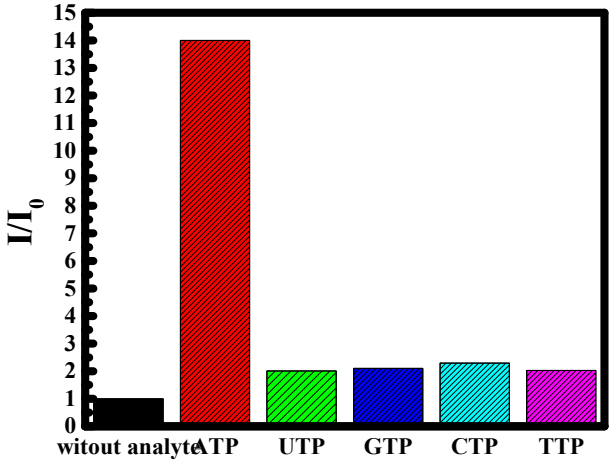

Figure 28: Ratio of emission intensity (I/I_0) of DMPC nanocapsule in the presence of ATP, UTP, GTP, CTP and TTP.

The analogous have similar structure to that of ATP, with the only difference is observed in the nitrogenous base. This suggests that there is a specific mode of interaction that occurred between ATP and nanocapsule.

The proposed method was followed to estimate the concentration of three samples. The results are summarized in Table 5.

Table 5: Recovery results of the method

	Theoretical Concentration (μM)	Experimental Concentration (μM)	Recovery (%)
Unkown 1	45	50	111
Unkown 2	70	71.755	102.5
Unkown 3	90	78.325	87.02

Finally, in order to investigate the photostability of the nanocapsule prepared, the emission intensity was measured within 1 hour every 5 minutes with/without ATP as shown in Figure 39.

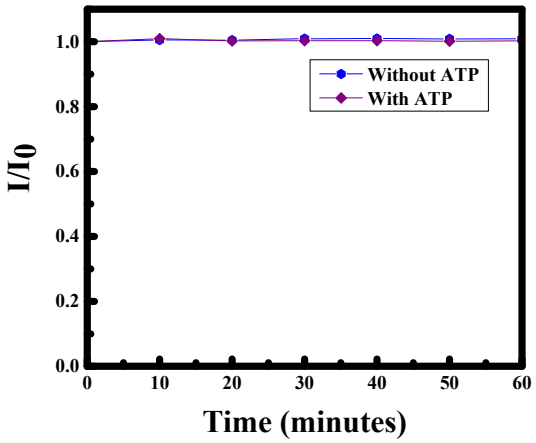

Figure 29: Plot of I/I0 of curcumin functionalized nanoparticles with time in the absence and presence of ATP.

It was remarkable that, the I/I_0 signal was found to be stable during one hour, indicating that our sensor is quite stable during time measurement.

4.3. Conclusion

Encapsulation of fluorophore using polymer can result in improvements of the fluorophore intensity. In the presented study, it was demonstrated that the emission intensity of curcumin was enhanced as the concentration of the ATP molecule increased and a blue sift was observed. This boost in curcumin's intensity could be explained by the aggregation of curcumin on the liposomal core, while the ATP molecule is expected to be adsorbed on the surface of nanocapsule by electrostatic interaction. The linear fit of I/I_0 emission intensity of the DMPC nanocapsule versus ATP concentration was well established. The increasing linear correlation allows the calculation of LOD (0.11 μM).

CONCLUSION

In the present work, different nanocapsules were synthesized based on self-assembly procedure for drug delivery and sensing targets. The three prepared DMPC capsules (N1, N2 and N3), were analyzed after curcumin being encapsulated within the core of the liposome. Nanocapsules were characterized using Scanning electron microscopy (SEM), Dynamic Light Scattering (DLS) and X-ray Diffraction (XRD). The morphology of the DMPC capsules show spherical and non-uniform spherical particles in N1 capsule. However, by increasing the coating layer to the surface of the liposomal curcumin, as the case in N2 and N3, more uniform and smaller size spheres appeared. Dynamic light scattering gave the particle size as 207 nm, 297.2 nm and 240 nm for N1, N2 and N3 respectively. Although, DLS results demonstrated higher particle size than SEM results, but the resulted DLS measurements could be attributed to the aggregation of the small spheres in the different of capsules. Furthermore, the interaction between the added polymer and the encapsulated curcumin was verified depending on XRD results. XRD peaks demonstrated broad patterns for the nanocapsules. Hence, the crystallinity property of curcumin was lost and changed to amorphous form. Further spectroscopic characterization such as UV-visible and Fluorescence were conducted for more understanding. UV-visible and emission fluorescence spectroscopic measurement manifested higher intensities in N3, this proved and that curcumin was more encapsulated in N3 capsule. Additionally, drug loading and encapsulation efficiency were calculated, the encapsulation efficiency of N1 was 92.407 %, whereas N2 and N3 had 87.22 % and 85.788 % values respectively. The less loss of curcumin in N1 is attributed to the less washing steps during preparation. However, N2 and N3 had more washing steps. The release of curcumin was shown to be less in N3 capsule, where N1

manifested higher release. Thus, the rate of curcumin's delivery decreased by attaching more layers on the surface. The drug release profile illustrated higher release at pH 4, in the three studied capsules.

The mixture of DMPC and DPPC liposome was synthesized at three various ratios, DMPC: DPPC. The synthesized capsules were analyzed by applying spectroscopic techniques, also the morphology was conducted by using SEM and DLS. In 50:50 ratio uniform spherical particles appeared, while by varying the ratios of DMPC: DPPC, different shapes were found, since DPPC longer hydrophobic chain than DMPC. The release of curcumin was high in 75:25 ratios, where it demonstrated the lowest release in 25:75. Though, the longer acyl chain in DPPC results in more curcumin's encapsulation. On the other hand, pH 4 exhibited the highest released curcumin's concentration on each ratio.

Curcumin has proved its massive function to act as florescent transducer. Thus, we have established Curcumin encapsulated in DPMC based liposome to test its ability to act as optical detector for ATP molecule. After the study was carried out, an increase in the emission intensity with a blue shift was observed as the ATP concentration increased. The detection limit was found to be 0.11μM. It was suggested that the positive charge of ammonium group in the polymer molecule could interact with the negative charge phosphate group through electrostatic interaction.

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