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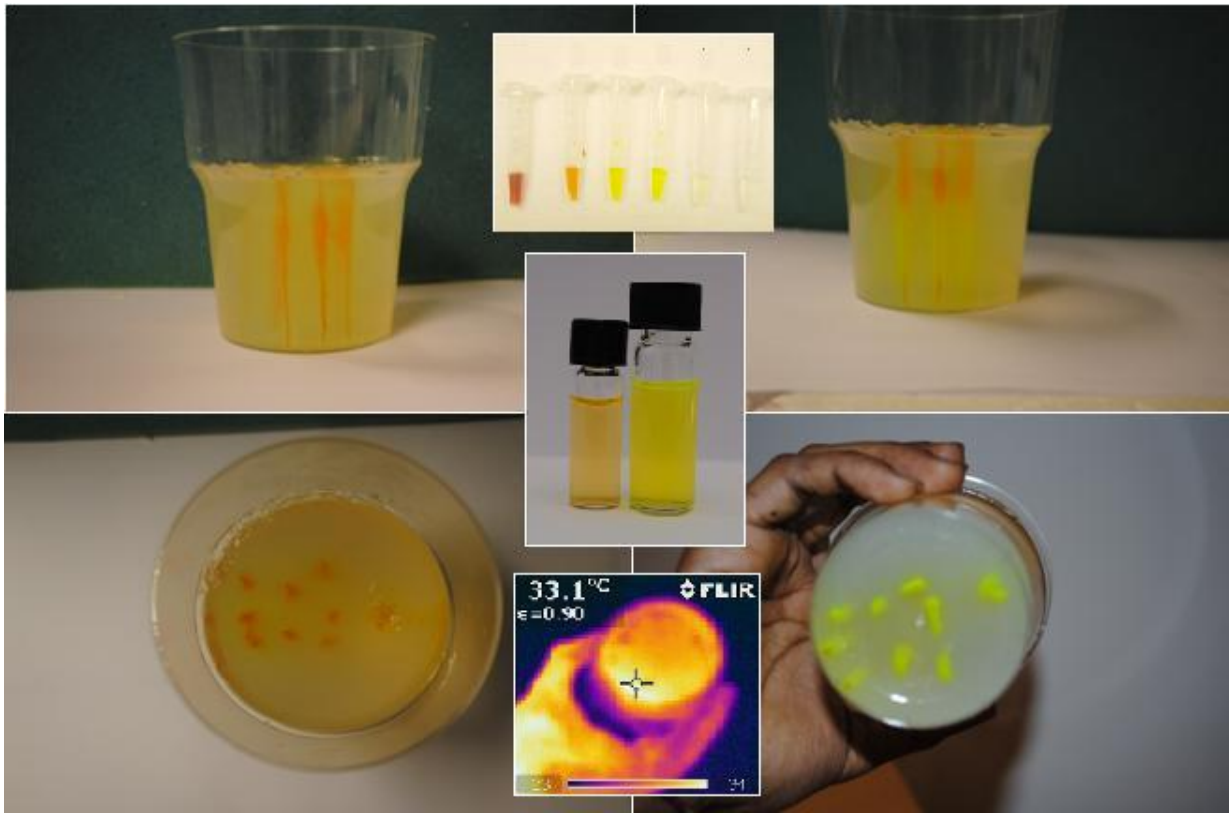

Application of hyperthermia for localized drug release from thermosensitive liposomes

Master's Thesis in Biomedical Engineering

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Chalmers Reproservice/ Department of Signals and Systems
Göteborg, Sweden 2010

Abstract

The use of Nanotechnology offers new possibilities to meet various challenges in the field of medicine, including cancer treatment. Chemotherapy is the widely used clinical procedure to treat cancer. The biggest challenge of chemotherapy treatment is to target the drug to the actual tumor site without damaging surrounding healthy tissues. Bimodal cancer treatment approaches can be a useful tools to meet this challenge. Hyperthermia is a promising technique that involves damaging or killing the cancer cells by exposing the tissue to high temperature. Liposomal drug carriers are used for releasing the drug at the targeted therapeutic site. The temperature caused by hyperthermia setup can be used as a stimuli for thermosensitive liposome to release the drug. The combination of hyperthermia and the thermosensitive liposmal drug delivery is promising and has been investigated by several researchers.

This thesis project aims at investigating the performance of a hyperthermia setup together with a model system for liposome based drug delivery. Thermosensitive liposomes were prepared by using dipalmitoylphosphatidylcholine (DPPC) and a fluorescent marker dye, carboxyfluorescein (CF) was encapsulated inside the liposomes using extrusion and gel filtration techniques. The size distribution of the liposomes was determined by utilizing the nanoparticle tracking analysis (NTA) technique. Fluorometric analysis was employed to characterize the release of CF from liposomes. Carboxyfluorescein is capable of self quenching, such that it fluoresces only when sufficiently diluted and it does not fluoresce when it is encapsulated at a high concentration. Thus the release of the carboxyfluorescein is associated with a higher fluorescence signal. CF-loaded thermosensitive liposomes were injected into an agar phantom in a certain array of columns and heated with the hyperthermia setup. The fluorescence obtained in the liposome array in three dimension depicted the local temperature rise caused by the hyperthermia system which triggered the release of carboxyfluorescein from the liposome.

Keywords: Bimodal cancer treatment, Hyperthermia, Thermosensitive liposomes, Carboxyfluorescein, Fluorescence

Acknowledgements

I would like to express my deepest gratitude to Sofia Svedhem and Hana Dobsicek Trefna, my thesis supervisors, for their helpful guidance, suggestions and for being patient while answering my questions. They have been a constant source of advice and wisdom over the last few months while I completed my thesis. I would also like to thank all the stuffs at biological physics group and bioelectromagnetics group at Chalmers.

Göteborg September 2010
Didarul Bahar Bhuiyan

Contents

List of Figures

1 Introduction

Advancement in nanotechnology has a promise to play a vital role in developing target specific drug delivery system for various diseases. Cancer is a disease, which is one of the leading cause of death in the present world. In a statistics presented by World Health Organization, it is shown that, the number of death due to cancer is projected to increase 47% from the year 2007 to 2030 (from 7.9 million to 11.5 million deaths), all over the world [20]. The number of cancer patients are predicted to increase rapidly in the coming years and the development of better procedures for cancer treatment is one of the prime ask in todays healthcare sector. Cancer is the disease which causes a group of cells to divide very fast and grow out of control. The word cancer is used for many different diseases, but all of them affects the body by the same way, the abnormal growth of cells. Current cancer treatment involves in chemotherapy, radiation therapy, surgery. Chemotherapy involves in the treatment of cancer by the injection of free drug into the blood stream. The drugs used for chemotherapy are strong and thus they kill any cell in the body that is growing fast, no matter what it is a cancer cell or a healthy cell. The efficacy of the chemotherapy is reduced by the lack of ability of the drug to reach their therapeutic site of action. Only a small part of the total drug actually reaches the tumor site and the rest is distributed to the whole body during chemotherapy. And as a result it damages the healthy organs or tissues surrounding the tumor. To solve this problem, liposomal drug carriers have been used for the delivery of cancer drugs via the blood stream. Liposomes theoretically fulfill the four basic requirement for a drug carrier - retention of drugs, evasion from the immune system of the body, targeting the specific therapeutic site and finally releasing the drug [9].

Liposomes are spheres with a lipid bilayer boundary. Liposomes are common drug carriers and they are capable of entrapping water soluble solutes in the interior volume, or hydrophobic drugs within the lipid bilayers. These vesicles are of 50 nm to 200 nm diameter in size. They form spontaneously when certain lipids are dispersed in aqueous medium. They are biodegradable, biocompatible and they do not evoke immune response [28]. The release of the content inside a liposome can be triggered by several chemical and physical stimuli. The surrounding pH, temperature and pressure can be such stimuli which can trigger the drug delivery. Among these, temperature is a very good external factor which is easier to control from outside the body. The flexibility of controlling the local temperature of the body by the external means, makes it easier to deal with the temperature sensitive drug delivery systems. The mechanism behind the triggered drug release from thermosensitive liposomes is the temperature induced membrane instability at the phase transition temperature (T_m) of the lipid. The phospholipid membranes undergo phase transitions, such as gel-to-liquid crystalline transition. In the gel phase of the lipid, the hydrocarbon chains are fully extended and closely packed. On the other hand, in the liquid crystalline phase of the lipid, the hydrocarbon chains are randomly oriented and fluid. Thus the phase transition temperature for the lipids is defined as the temperature required to induce a change in the lipid physical state from the ordered gel phase to the disordered liquid crystalline phase [30]. Generally, the lamellar structure of liposome membrane at the gel-to-liquid-crystalline transition phase is fairly loose and porous. And this phenomenon is utilized during designing the temperature sensitive liposomes. When a liposome is heated at the phase-transition temperature, it is supposed to release its content as it changes from the closely packed more ordered form to a randomly oriented disordered form.

Hyperthermia is a cancer treatment procedure that involves in damaging or killing the

cancer cells by exposing the tissue to high temperature. This therapy is combined with other existing cancer therapies such as radiation therapy or anti-cancer drugs to increase the effectiveness. The hyperthermia treatment elevates the temperature in the tumor volume above 42-43°C for a certain period of time and the system is supposed to keep the temperature at the normal physiological level for the surrounding tissues. It has been observed that the temperature above 42°C is directly cytotoxic to the cell [23]. The exact mechanism for this cytotoxicity is still not completely understood, but it has been hypothesized that, the protein denaturation caused by the heating plays the main role in the cell death. Observations depict that above 42°C, the programmed cell death, apoptosis is induced and above 45°C necrosis (the premature death of cell) is enhanced. For the mild hyperthermia treatment, the temperature is kept at 40-41°C in the tumor tissue. This temperature does not directly induce cell death, rather it enhances phenotypic changes which makes the tumor tissue to be more susceptible to the effect of other treatment types such as radiation or chemotherapic drugs [12]. Thus the local hyperthermia has the potential to play a vital role in enhancing the efficacy of the anti-cancer drugs in the specific site of tumor. The combination of temperature sensitive liposome and hyperthermia have been successfully examined by several research groups. Yatvin and Weinstein were the pioneers in this field who first described the formulation of liposomes in the late seventies which was composed of phospholipids having the gel to liquid phase transition temperature at 44°C [7]. Needham et al. have described the formulation of a thermosensitive liposome and combined it with mild hyperthermia to show its more significant effect than the free drugs in a human tumor model [21]. Lindner et al. have also described a new temperature sensitive liposome formulation to be used with hyperthermia and examined the effectiveness both invitro by using a fluorescence dye and in vivo by using animal models [9]. Morita et al. have also run similar experiments to investigate the efficacy of the drug loaded thermosensitive liposomes combined with local hyperthermia on rat tumor models [18].

The aim of this thesis was to investigate basic performance of a hyperthermia setup combining with model system for liposome based drug delivery to address the localized heating for site specific drug release. A fluorescent marker dye (carboxyfluorescein) was chosen as substitute for the real drug. Carboxyflurescein is capable of self quenching, such that it fluoresces only when sufficiently diluted, and it has previously been encapsulated inside liposomes at high concentrations. As the encapsulated carboxyflurescein does not show the fluorescence, the fluorescence signal was considered to be the indication of its release from liposome. The idea of the present thesis project was to locally release carboxyfluorescein loaded into thermosensitive liposomes inside an agar phantom with dielectric properties similar to muscle tissue and to look into the effectiveness of the heating system in three dimensions. The liposomes with the carboxyfluorescien inside, were injected inside the agar phantom in a certain array of columns and heated with the hyperthermia setup and finally the change of the color of the marker dye was observed to estimate the effectiveness of the system.

2 Background

In this chapter a brief overview of the different techniques used for the experiments are presented.

2.1 Microwave Hyperthermia

Hyperthermia Heating Technique

For elevating the tissue temperature, three conventional heating techniques are used for hyperthermia system: Electromagnetic heating , thermal conduction and ultrasound. The ultrasound technique provides better spatial and dynamic control and also a better control over the temperature distribution. But on the other hand it has the problem of having higher acoustic absorption at bone interface which might induce patient discomfort and frequent pain from bone heating. Thermal conduction is rather simple technique which involves hot balloons that are brought near to the tumor by inserting it through a natural opening of the body. But this technique is mostly used for the treatment of superficial tumors. Due to the disadvantages of the other two techniques and the for its versatility, electromagnetic technique is most frequently used.

On the basis of the method of energy delivery to the tissue, the electromagnetic applicators can be divided into two categories- external and interstitial. The interstitial one can be invasive while antennas are implanted within the tumor. If the antennas are inserted in natural openings of hollow organs such as urethra, rectum etc, it is called inter-cavitary. For the external heating, the non-invasive antenna arrays are placed on the surface of the body. The hyperthermia heating can be local, regional and part-body or whole-body heating [26].

Local Hyperthermia

Local hyperthermia is the most recognized hyperthermia treatment used for treating superficial tumor. Local hyperthermia increases the tumor temperature and as a result it increases the tumor perfusion and oxygenation. The electromagnetic energy is applied to the tumor volume by the applicators placed on the surface. A water bolus is used as a matching liquid for matching the electromagnetic properties between the applicator and the body. The schematic diagram of a typical local and interstitial hyperthermia system is presented in figure 2.1. The EM field is produced by the generator and applied to the tumor volume by the applicator. The thermometer connected to the PC takes the feedback of temperature at the site of application via the interstitial probes. The temperature probes are usually used invasively to measure the temperature at the tumor site. The probes should posses such properties that it does not interfere with the electromagnetic field. For the superficial tumors, probes are used on the surface of the body under the water bolus. But for the deep seated tumors, the probes are inserted into the tumor by the catheters. The typical operating frequency for the local hyperthermia is in the range of 400 MHz to 2.45 GHz which allows therapeutic depth of less than 3 cm [12].

Regional and part body hyperthermia

Regional hyperthermia is used for treating deep seated tumor inside the body. The main idea of regional hyperthermia is to concentrate the heat at the tumor site and to keep the temperature at the surrounding healthy tissue under the safety level. Most widely used

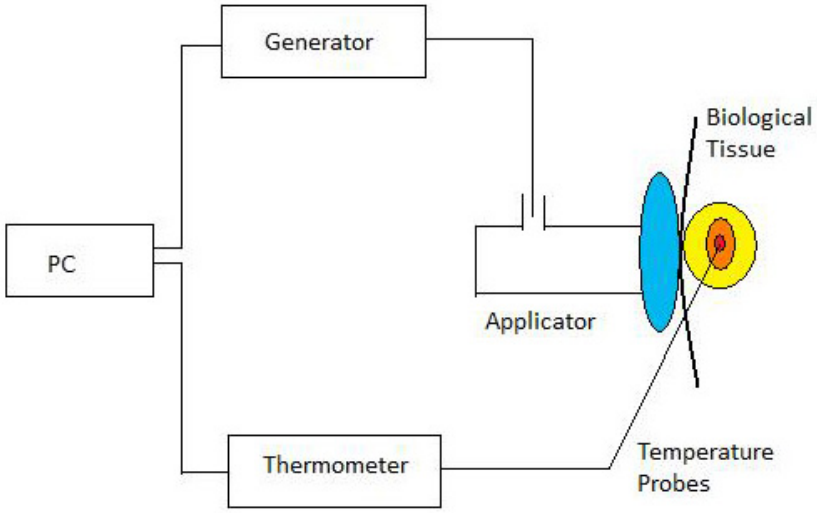


Figure 2.1: The schematic diagram of the typical system of a local interstitial hyperthermia system. Reproduced from [13].

regional hyperthermia setup includes a ring shaped array of antennas placed circumferencially surrounding the patient to heat the tumor. By changing the amplitude and the phase at the antenna feed points, the wave interference can be obtained. The penetration depth of the radiation wave depends on the working frequency. The typical range of operating frequency for regional hyperthermia is 70 to 200 MHz for the pelvic region and 434 MHz for head and neck region [12].

Whole body hyperthermia

For treating some special type of cancer such as carcinoma with distant metastases, it is required to achieve a steady state temperature of around 42°C in the whole body for typically one hour. And as a result the energy loss from the body is minimized as well as energy is introduced to the body from outside. Microwave and infrared radiation techniques are presently clinically used for whole body heating [12].

2.2 Phantoms

A new medical product needs to be tested on a non-living object before it can be applied to an animal. Thus, the phantom plays the role of non-living object for the testing. Phantom is a physical model that imitates certain characteristics of a biological tissue. By means of experiments using phantoms, it is possible to quantitatively measure and estimate the interaction between the biological body and the electromagnetic field.

The components of the biological tissue are affected in cellular and molecular level when they are exposed to electromagnetic fields. In the macroscopic level, for the simplicity of modeling, only two dielectric properties are considered to be involved permittivity and conductivity. Permittivity is the amount of resistance that is faced during formation of the electric field in vacuum. In other words, it determines the effect of polarization on the charged medium due to the applied field [17]. And the conductivity of the material is the measure of the ability of the medium to conduct electric current. It also describes the electrical losses in the material due to the electric current driven by the applied field.

The permittivity and the conductivity of the biological tissue is dependent on the applied frequency of the field. Generally, the water content of the tissue is the determinant of its dielectric properties. Muscle or skin tissues which have higher water content have significantly higher value of permittivity and conductivity than the tissue with lower water content such as fat or bone [25].

To achieve the desired properties within the phantom, different components are added to it. The amount of sugar and salt present in the phantom affects the permittivity and the conductivity of the phantom. But sugar has a greater effect on the permittivity whereas salt plays greater role in increasing the conductivity. Anna Gund and Stefan Lindqvist, in their master thesis report, has provided several recipes for preparing different phantoms mimicking different body tissues , such as breast fat, blood, skin, muscle etc, based on their dielectric properties [2].

2.3 Preparation of liposomes encapsulating a dye

Carboxyfluorescein dye

Carboxyfluorescein is the most commonly used marker dye to measure the rate of release of vesicle contents. This marker was chosen instead of the real drug to visualize the release of the content from the liposomes. Before using liposomes as potential drug delivery system, the rate at which entrapped drug molecules leak out under various experimental conditions is inquired. Carboxyfluorescein is the fluorescent dye, which is the most commonly used marker to assess the rates of leakage of water-soluble substances from liposomes. The molecular structure of carboxyfluorescein is shown in figure 2.2. Note that carboxyfluorescein is not water soluble in this form. See section 3.2 in the chapter Materials and methods.

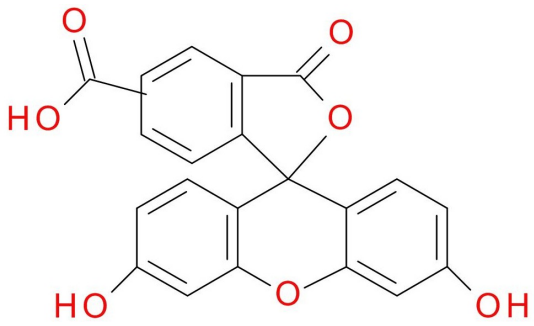


Figure 2.2: The molecular structure of 5(6)-carboxyfluorescein

Carboxyfluorescein has a property of self quenching; 97-98% of the fluorescence is quenched, when 5(6)-carboxyfluorescein is encapsulated in liposomes at 0.2 M as it is mentiond by Chen and Knutson [29]. This depicts that when carboxyfluorescein is encapsulated in liposome at a high concentration it fluoresces after the release as it reaches a low concentration. And this fluorescence signals can be measured with fluoremeter. Thus, the higher fluorescence signal obtained from the fluoremeter indicates a successful release of the carboxyfluorescein from the liposome. The excitation and emission wavelength for carboxyfluorecein is 492 nm and 520 nm, respectively [29].

Liposome Preparation

Liposomes are commonly used as models of biological membranes. In past decade liposomes have gained more frequent usage as a drug carrier due to its ability to reduce drug

toxicity and to deliver the drug at the site of action. Liposomes are classified into three classes according to the size and the number of bilayers such as Large multilamellar vesicles (MLVs), Small Unilamellamar Vesicles (SUVs) and Large Unilamellar Vesicles (LUVs). The unilamellar vesicles with diameters less than 100 nm are considered to be SUVs and more than that are considered as LUVs. The size of MLVs ranges from 100nm to 1μm and generally consists of five or more lamellae or boundary [24].

There are several ways to prepare liposomes. First a lipid suspension is formed by the hydration of dry lipid films. Hydration refers to the addition of an aqueous medium to the lipid film. Before hydration lipid films are formed on a surface and hydration helps them to become fluid and swell [1], which eventually detach from the surface to form Large Multilamellar Vesicles [5]. Finally, the size of these large vesicles are reduced by applying sonic energy with sonication or by applying mechanical energy by following extrusion procedure. The properties of the liposomes depend on the composition of the lipid. But the preparation procedure is the same irrespective of the composition. And the procedure includes the preparation of the lipids for hydration, hydration with agitation and finally sizing to a homogeneous distribution of the vesicles [6].

Extrusion technique

Extrusion is a procedure that is applied for producing vesicles. In this procedure, a solution with the vesicles with several layers of lipids, which are also known as multilamellar vesicles are forced through the pores of a polycarbonate filter membrane for several times to obtain the required vesicle. A syringe-based plunger system or pressured gas [15] is used for forcing the multilamellar vescile solution through the pores. The pores of the polycarbonate membranes are of long and thin cylindrical shape. The length of the pores is about 6 μm, and the diameter of the pores is about 100 nm or smaller [10]. Multilamellar vesicles are pushed through polycarbonate membrane filters at least 10 times, which gives small unilamellar vesicles with size comparable to the pore size. The size of the vesicles depends on the size of the pores. The extrusion pressure applied to the vesicles also affects their size. An increased extrusion pressure produces slightly smaller vesicles [19]. The extrusion procedure should be operated above the gel-fluid transition temperature as the fluidity of the lipid through the pores decreases below the transition temperature [22]. The assembly of an extruder is presented in figure 2.3.

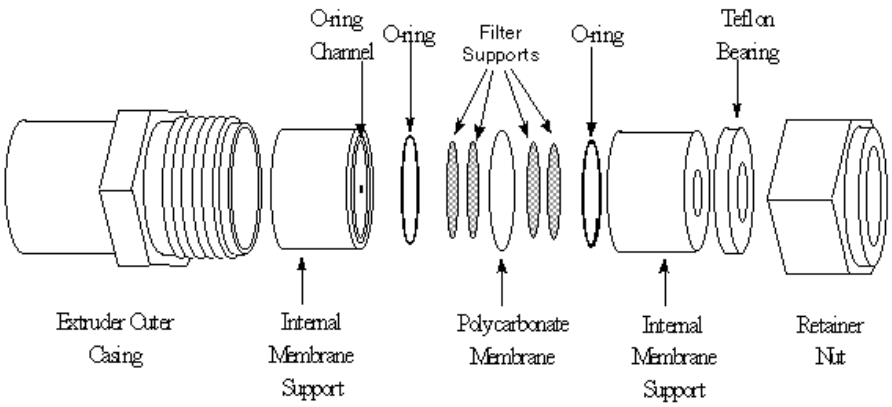


Figure 2.3: Example of an extruder for a liposome preparation (Mini extruder, Avanti Polar Lipids Inc.)

2.4 Methods for liposome purification and characterization

Gel Filtration

To purify liposomes, several techniques can be used such as chromatography and gel filtration. Gel filtration is an efficient method to remove excess of low molecular weight compounds from liposome solution due to their large difference in size. The gel filter column consists of the porous matrix of a medium which is chemically and physically stable and inert. The material used for media includes peptides, proteins, protein complexes etc. depending on the sample and the required flow rate. The column is equilibrated with buffer which fills the pores of the matrix and the space between the particles. The most significant advantage of using gel filters over other separation procedure is that the conditions can be varied according to the sample and it is not required to further purify the sample after the separation for the doing the analysis as for other methods like ion exchange chromatography, the molecules bind with the chromatographic medium and it requires further purification. Thus, gel filtration is a robust technique which is suitable for biomolecules that are sensitive to change of conditions such as pH and ion concentration [8]. A schematic of the filtration procedure is presented in figure 2.4. The filtration process by the gel filter. The solution containing the large and the smaller particles are introduced to the filter column. With time, the larger molecules comes down to the bottom of the column through the column medium due to gravity. Finally the larger molecules are separated while the smaller particles are still in the column.

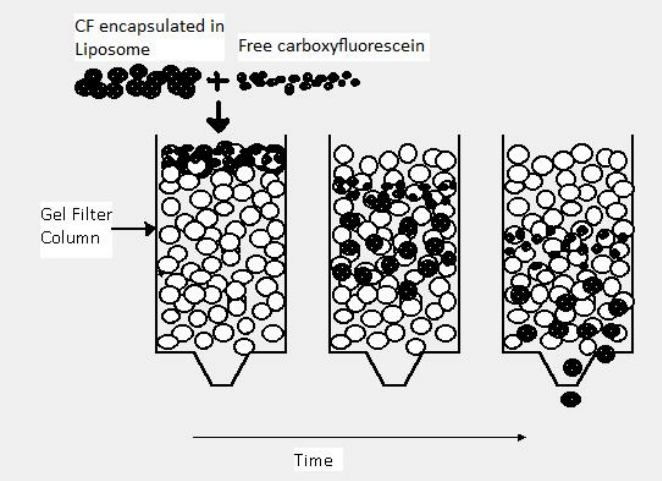


Figure 2.4: The filtration process by the gel filter. Adapted from [8]. Exemplified for the kinds of liposomes prepared in the present project.

Fluorometry

The term fluorescence refers to the molecular absorption of light at a wavelength in a certain substance and an instantaneous emission of it at a different wavelength, usually at a longer wavelength. The fluorescent substances have two characteristic spectra which are unique for each substance- the excitation spectrum and the emission spectrum. The excitation spectrum refers to the variation of intensity of emitted light with the wavelength of the absorbed light and the emission spectrum is the variation in intensity of absorbed light with the wavelength of the emitted light. Fluorometry is the procedure for measuring the fluorescence and the instrument used for the purpose is called fluorometer. It involves in generating a wavelength of light which excites the substance and then it transmits the

wavelength of light emitted and finally measures the intensity of the transmitted light [4].

Fluorometers can be of two types- filter fluorometer and spectrofluorometer. The filter fluorometer uses optical filter to provide specific excitation and emission wavelength. The filter can be changed mechanically for different optical filter configurations. The spectrofluorometer uses an excitation monochromator and an emission monochromator instead of optical filters. With the spectrofluorometers, substance can be scanned over a wide range of wavelengths. The filter fluorometer has a light source which produces the light at the wavelength range for exciting the measured substance. The light source includes xenon lamps, mercury vapor lamps, lasers, LEDs. Light generated by the source passes through the excitation filter which transmits only the wavelengths specific to the excitation of the measured substance and blocks all other wavelengths. Then the light passes through the sample and excites it and the emitted light is passed through the emission filter. Cuvettes are usually used to hold the sample which allows the absorption and emission energy to pass through. Cuvettes are commonly made of borosilicate or quartz glass or plastics which can pass the selected wavelength of light. The emission filter screen outs the background stray light and allows only the wavelength of light specific to the substance. The emitted light is then measured by the detector and displayed. The principle of the fluorometer is presented in figure 2.5. Photomultiplier tubes or the photodiode are often used as a light detector [16]. Finally the light detector provides the digital output.

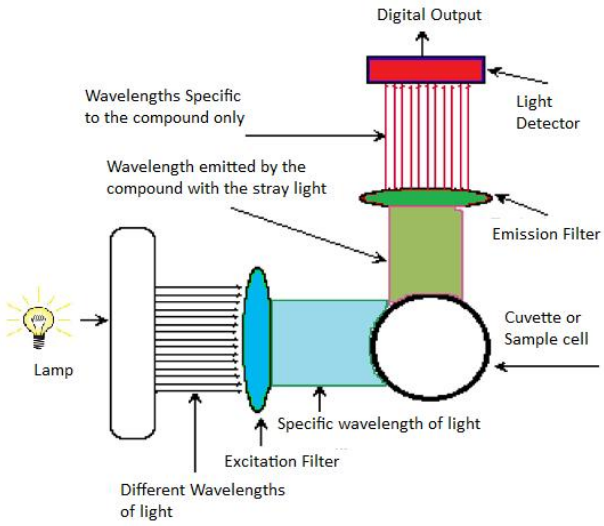


Figure 2.5: The principle of fluorometer. Adapted from [11].

Nanoparticle Tracking Analysis (NTA)

Nanoparticles are used in various application in several fields, specially in pharmaceutical industries. With the increased application of nanoparticles in various fields, the necessity of analyzing nanoparticles has become more and more important. Nanoparticles have been analyzed for the particle size or the size distribution with number of techniques for long years, such as electron microscopy, photon correlation spectroscopy, dynamic light scattering, Fraunhofer scattering, single particle detection techniques, optical microscopy [3]. Recently a new technique, known as nanoparticle tracking analysis, has been developed which allows to visualize and analyze the nano-particle in liquid by correlating size with brownian motion [27]. The nanoparticles are analyzed in real time by capturing the real

time image by a charged coupled device (CCD) camera. Each particle are detected by using an image analysis program which allows to visualize and analyze each particle separately.

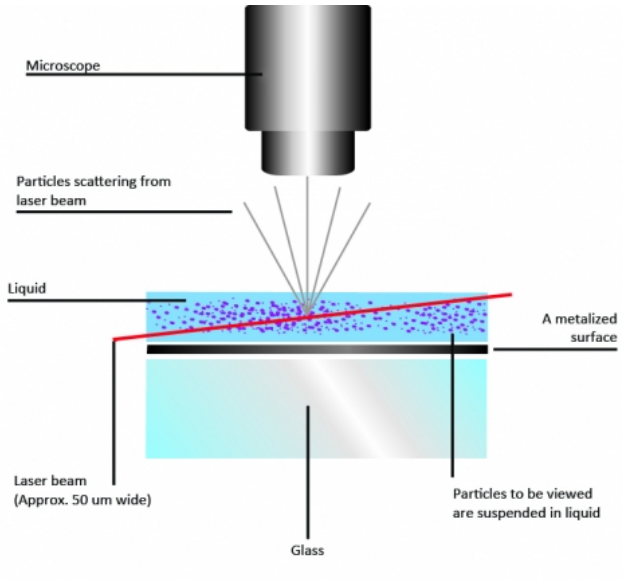


Figure 2.6: The principle of NTA. Adapted from [27].

The NTA technology is composed of a laser device contained in a metal box which produces a finely focused laser beam to the chamber where sample is placed. The sample chamber is specially designed and has an optical window through which the particles can be observed with the help of microscope. The sample is placed into the chamber by syringe. The laser beam produced by the device passes through a prism edged optical flat which refracts the beam at the interface between the optical flat and the liquid sample layer placed over it. The particles in the liquid sample that pass through the beam path can be observed by the microscope as a small point of light moving rapidly due to the brownian motion. The principle is depicted in figure 2.6. The microscope is connected to a CCD camera which is used to capture the video of the scattered particles moving under brownian motion. The NTA program simultaneously identifies and tracks the centre of each particle on a frame-by-frame basis throughout the video, which allows the program to determine the mean squared displacement for each particle for the visible trajectory.

Particle movement rate is related to the size of the particle and the viscosity of the liquid medium through which it is moving. It is also dependent on the temperature but the particle density does not influence the rate of movement. NTA system utilizes this idea to determine the particle size and the size distribution. Knowing the values of the diffusion coefficient, Boltzmanns constant, temperature and viscosity , the hydrodynamic radius of the particle can be determined by using the Stokes-Einstein equation[14].

$$D = \frac{K_B T}{6\pi\eta r_h} \qquad (1)$$

where,
D = Diffusion Coefficient,
K_B = Boltzmann's constant,
T = Temperature,
η = Viscosity,
r_h = Hydrodynamic radius

3 Materials and methods

3.1 Preparation of Phosphate buffer

Phosphate buffered saline (PBS) was prepared dissolving tablets (Sigma Aldrich, product ID P4417) in water. All water used was purified using MilliQ purification system. THe pH of the solution was verified. Dissolving of one tablet in 200 mL yielded 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride at pH 7.4 at 25°C.

3.2 Preparation of Carboxyfluorescein stock solution

282 mg (0.75 moles) of 5(6)- Carboxyfluorescein (CF) (Sigma Aldrich, product ID 21877) was dissolved in 1.5 mL of 1 M NaOH solution (1.5 moles) to make it water soluble. The pH was adjusted to 7.2 and the volume was adjusted to 2.5 mL by the addition of water to give 2.5 mL of 300 mM CF stock solution.

3.3 Preparation of Lipid film

12 mg of dipalmitoylphosphatidylcholine (DPPC) (Sigma Aldrich, product ID P4329) was weighed out into a small glass vial and dissolved in 2 mL of chloroform and placed in a round bottom flask. Chloroform was removed under a stream of nitrogen gas at around 1 bar pressure in the fume hood. The round bottom flask was kept under vacuum overnight to remove solvent residues and the obtained lipid film was stored in refrigerator.

3.4 Lipid vesicle preparation

Prior to extrusion, the lipid film was hydrated with the CF stock solution at elevated temperature ($>T_m$ of DPPC) and vortexed. The lipid suspension was extruded using a Mini-Extruder (Avanti Polar Lipids Inc.) with a filter pore size of 100 nm. For assembling the extruder, the internal membrane supports were placed on a flat surface with the o-rings facing up and pre-wetted with NaOH solution so that the filter support can adhere to it. After assembling the extruder, the assembly was placed on the heating block on a hot plate and the temperature of the block was maintained over the phase transition temperature of the lipid (∼60°C). The hydrated sample was loaded in a syringe and placed into one end of the extruder shown in figure 3.1. An empty syringe was placed on the other end and pressurized gas was used for pushing the solution back and forth between two syringes for 11 times to obtain the extruded liposomes.

3.5 Liposome purification

Liposome samples were separated from the free carboxyfluorescein using NAP-5 gel filter columns (GE Healthcare), eluting with PBS. For further analysis, samples of 0.1 mL fractions from different stages of the gel filtration were collected separately. Liposome samples were pooled from different fractions of gel filtration into a single sample to get a more uniform solution. To get rid of the free carboxyfluorescein more accurately, the sample was filtered twice with the gel filter column.

3.6 Liposome Characterization

The liposome size distribution and concentration at different stages of the gel filtration were evaluated by nanoparticle tracking analysis. Samples were prepeared by adding 2 μL

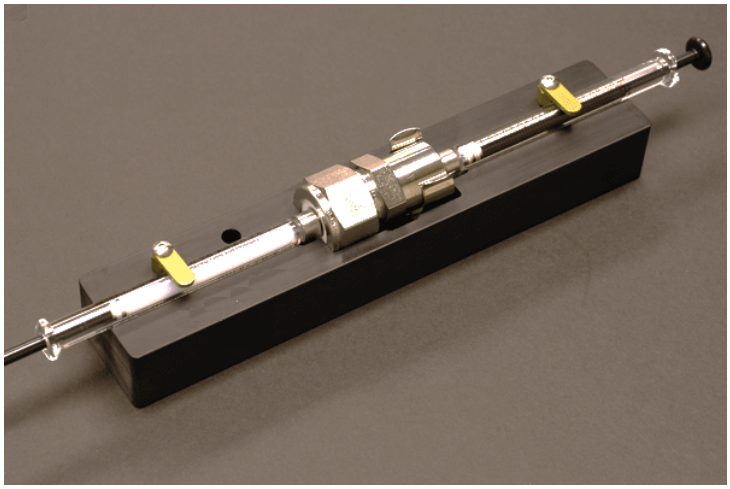


Figure 3.1: The arrangement of the extruder and the syringe assembly

of liposome sample into 1 mL of PBS buffer. Latex samples (140 nm, reference microparticle, Sigma Aldrich) were also analyzed with the nanoparticle tracking analysis system as reference. The flow chamber of the NTA system was cleaned by rinsing the prism and the metal holder with ethanol and water and dried with a stream of nitrogen gas. Then the sample was injected into the inlet of the flow chamber by syringe and temperature reading was noted. The particles scattering from laser beam was observed by the microscope which is connected to the camera. Video was captured and processed with the nanosight NTA analytical software program by setting the brightness, gain, blur and detection threshold values.

CF solutions were analyzed at six different concentration ranging from 300 mM to 0.003 mM by the fluorometer using a 1 cm square cuvette. Samples were prepared by adding 40 μL of the CF solution into 2 mL of water. Samples were also prepared by using PBS buffer instead of water and analyzed with fluorometer (Photon Technology International) which was connected to the computer through the PTI Felix32 software. To prepare the liposome sample for fluorometric analysis, 10 μL of liposome solution was diluted in 2 mL of PBS buffer. Sample solutions were heated with the heating system connected to the fluorometer and emission spectra were obtained at different temperature, starting from 25°C to 65°C to visualize the release of the carboxyfluorescein from the liposomes. The temperature inside the cuvette was measured using a thermocouple. Similar experiments were performed using Glycerol and Polyethylene Glycol (PEG) instead of PBS buffer to compare the release of carboxyfluorescein from the liposome in different media.

3.7 Muscle phantom preparation

Muscle phantom was prepared by mixing 40% sugar (Dan Sukker), 0.35% sodium chloride (Falksalt) and 4% technical agar into water. All the percentages of the ingredients are in percentage of total volume. The mixture was then boiled using an electric heating plate and poured into the mold to provide the required shape and kept in the room temperature overnight for cooling.

3.8 The hyperthermia system

The hyperthermia system used for this thesis has an effective output power of 100 W and an operating frequency range of 300 MHz to 1 GHz. The system consists of 12 identical

channels which are driven by a common excitation signal. The excitation signal is generated by the signal generator and divided into 12 paths by a power divider. All the system components are interfaced with a computer through data acquisition module. The applicator is based on a ring shaped antenna array consisting of 12 triangular antenna element as shown in figure 3.2 [13]. The antenna array is submerged in the water bolus which is used as a matching liquid to match the electromagnetic properties between the phantom and the antenna. The phantom is placed inside the water bolus such that the central axis of the cylindrical phantom coincides with the central axis of the circular antenna array. The bottom surface of the phantom was placed on a plastic support so that it remains at the same level as the bottom point of the antenna. The water level was kept just above the top surface of the phantom. Optical probes were inserted into the phantom to measure the temperature at different location. Optical probes were used as they do not interfere with the electromagnetic signal. The system was ran at 434 MHz at the maximum power. After heating, the phantom was taken out and thermal images were taken using IR camera (FLIR 650) for using as reference.

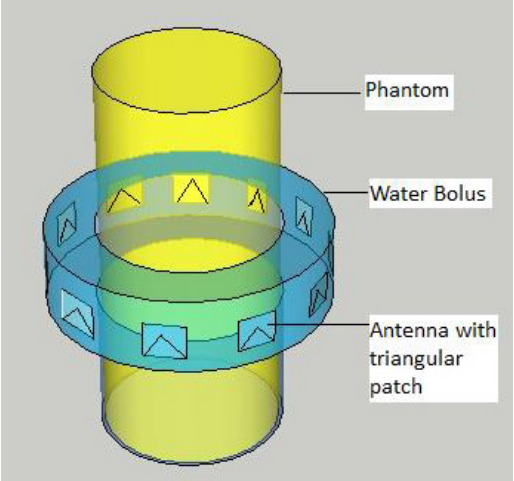


Figure 3.2: Three dimensional visualization of twelve antenna array placed surrounding the phantom. Reproduced from [13].

4 Results

4.1 Design of the experimental setup

The first idea of the experiment was to have liposomes distributed in the whole phantom and then to heat it with the hyperthermia system to visualize localized release of CF. However to fill up the cylindrical muscle phantom of diameter 10 cm and hight 10 cm, which gives a volume of 785 mL, a large quantity of liposomes were required. Moreover with the gel filter, in each batch, only 0.5 mL of liposome could be separated, which makes the large quantity of liposomes needed to fill up 785 mL of phantom unrealistic.

The next idea was to insert a transparent plastic tube filled with liposomes, inside the muscle phantom as shown in figure 4.1 and heated with the hyperthermia system. Thermal probes were inserted at three different heights of the tube to get the idea of elevation of temperature at different hight. The system was stopped after the thermometer reading reached 41°C and liposome sample was taken out of the plastic tube and analyzed with fluorometer. The high fluorescence signal obtained from the heated sample ensured that the hyperthermia system was able to induce the release of CF from the liposomes.

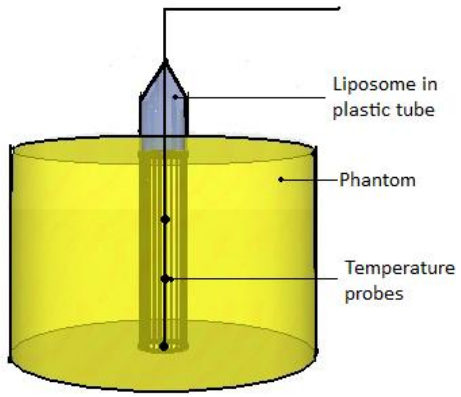
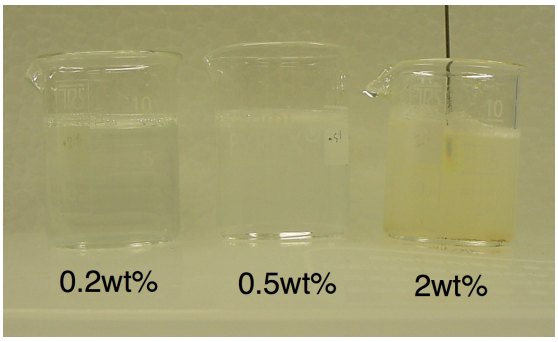


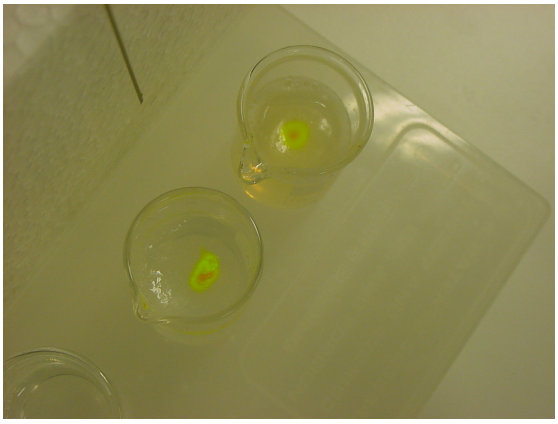
Figure 4.1: The experimental set up with a plastic tube placed inside the muscle phantom.

However, from this experiment, localized release from liposome could not be demonstrated. Thus the next idea was to demonstrate the local release in small scale. Three different gels were prepared containing three different weight percentage of agar- 2 wt%, 0.5 wt% and 0.2 wt%. by adding them each in 10 mL PBS buffer. Liposomes were injected into the agar gel phantoms shown in figure 4.2. The bright yellow (fluorescent) area is due to remaining free CF in the liposomes, and was prevented in later experiments by improving the gel filtration procedure. A more purified sample was used in figure 4.3 where the agar gel phantom with liposome coloumn was heated with the heating plate. THe phantom was heated from below. Liposome were stable in the agar gels, and the gels were dense enough to prevent liposome diffusion. From the three gel phantoms, the one with 0.2 wt% agar was most transparent and the liposome column could be easily viewed through it.

Finally for the demonstration of localized release of CF, instead of having liposome all over the muscle phantom, array of liposome columns were prepared in a phantom made by adding 0.2 wt% of agar gel in 200 mL of PBS in a plastic cup. This phantom is referred as Liposome phantom in this thesis. Liposome columns were injected with needle in an array as shown in figure 4.4.

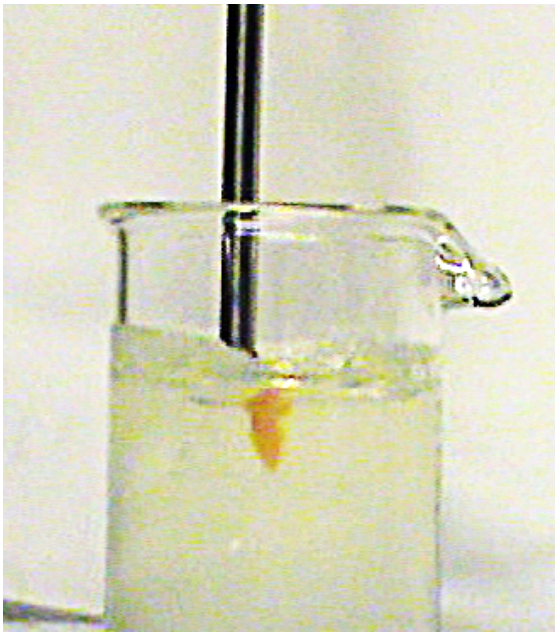


(a) Gel with different weight percentages of agar.



(b) Injection of liposomes into agar gels.

Figure 4.2: Injection of liposomes in different agar gels



(a) Liposome column before heating.

(b) CF release after heating with hot plate.

Figure 4.3: Release of CF from liposome column heated by hot plate from below.

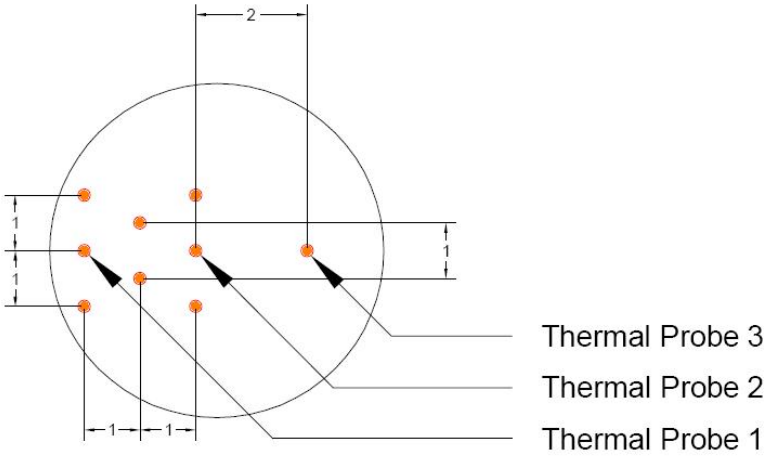
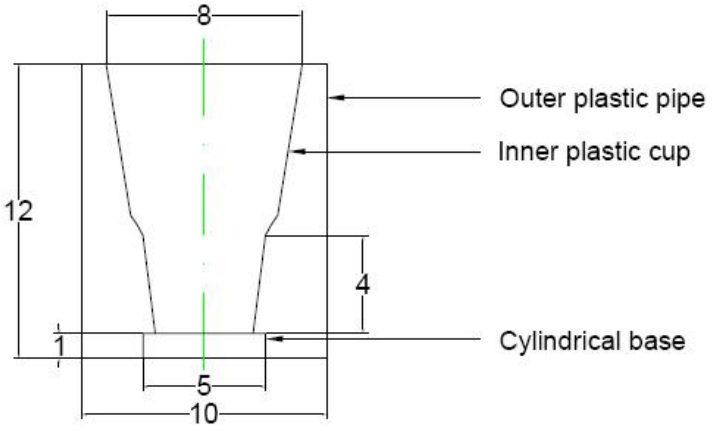


Figure 4.4: Top view of the array of liposomes injected in the agar gel.



(a) Front view: Design of mold for the muscle phantom.

(b) Molded muscle phantom.

Figure 4.5: Design and molding of muscle phantom.

The muscle phantom was molded with cavity to place the Liposome phantom in it . A plastic pipe of 12 cm height and diameter of 10 cm was used as the container to provide the outer shape of the phantom. The taper shaped plastic cup which contained the Liposome phantom had a hight of 10 cm and its upper diameter was 8 cm and at the bottom it had a diameter of 6 cm. This cup was placed at the center of the pipe, 1 cm above from the bottom surface by placing it on a plastic cylinder of diameter 4 cm and hight 1 cm. The design of the mold and the molded muscle phantom is presented in figure 4.5.

4.2 Characterization of CF

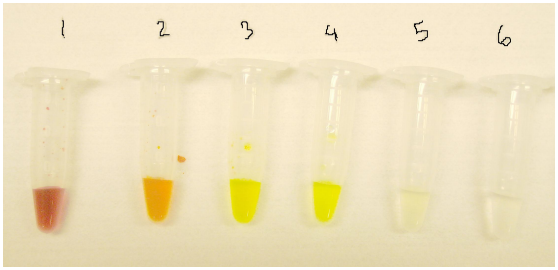
CF is known to self quench at concentration higher than 200 mM [29]. This phenomenon is demonstrated in figure 4.6. Figure 4.6a contains the CF solutions at six different concentration ranging from 300 mM to 0.003 mM. Comparing tube 1 with tube 3, it can be clearly visible that CF is self quenched at 300 mM concentration and it fluoresces after it was diluted. This phenomenon is further verified by analyzing the samples with a fluorometer. From the obtained graphs, the highest emission signal was observed for a concentration of 0.3 mM.

It is to be noted that the excitation wavelength was not same for all the samples. The excitation wavelengths are given in the table 4.1.

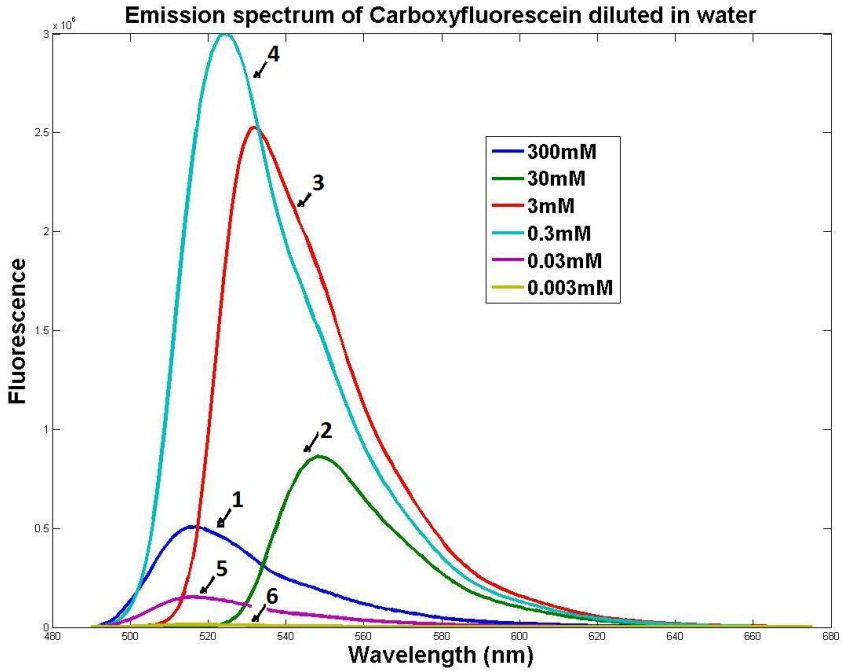
Solution	Excitation wavelength in nm (for solution diluted in water)	Excitation wavelength in nm (for solution diluted in PBS)
1	532	535
2	520	524
3	512	470
4	495	495
5	492	492
6	492	492

Table 4.1: Excitation wavelengths used for different samples.

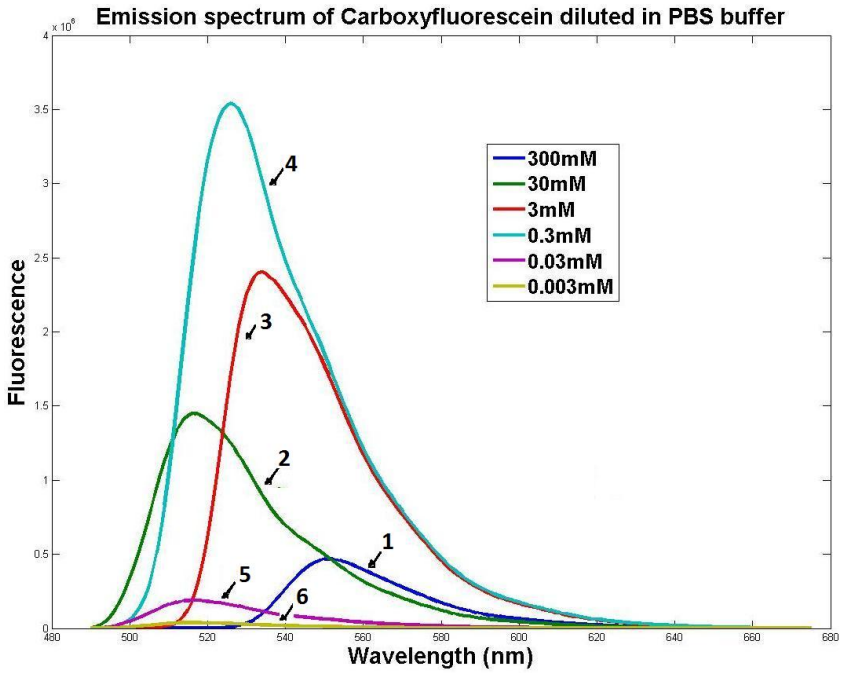
Under the conditions used, the peak of the curve was observed to shift, though the



(a) CF solutions at six different concentrations.



(b) Emission spectra for CF diluted in water.



(c) Emission spectra for CF diluted in PBS.

Figure 4.6: Characterization of CF solutions at different concentrations and in different media.

shape and pattern of the emission spectra were similar for CF diluted in water and in PBS buffer.

The effect of elevated temperature on the fluorescence of free carboxyfluorescein was analyzed. Under the conditions used, the peak value of the fluorescent count decreased at a negligible range with the increase of temperature and the peak has also shifted slightly towards higher wavelength with the increase of temperature shown in figure 4.7. Thus it was concluded that the temperature change does not affect the fluorescence of the free carboxyfluorescein.

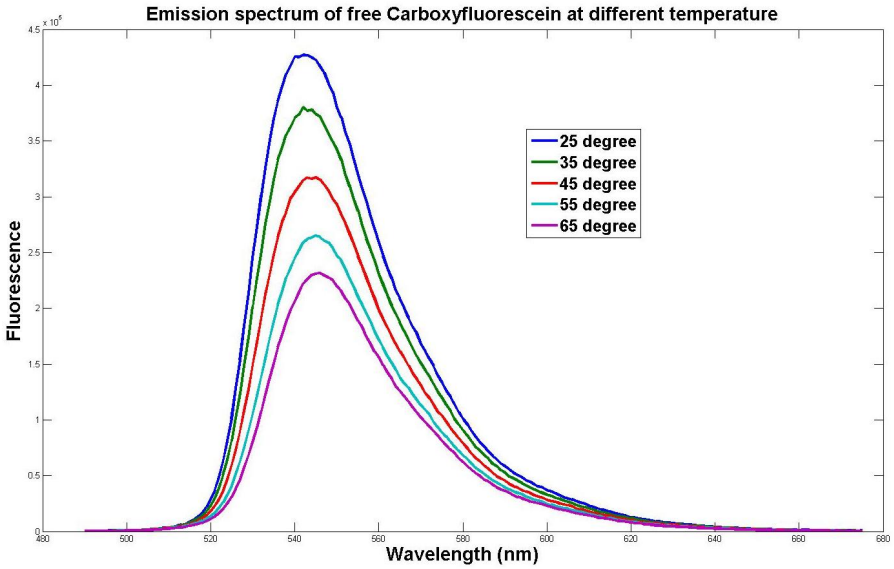


Figure 4.7: Emission spectra for free CF at different temperatures.

4.3 Characterization of liposomes

Size determination

The liposome samples were analyzed to determine the size distribution and particle concentration after gel filtration. Samples were collected from different stages of gel filtration. It was predicted that the fractions from the earlier stages of gel filtration would contain more liposomes than the fractions from the later stages. However the particle concentration obtained from the nanoparticle tracking analysis as shown in table 4.2, does not comply with the prediction, rather it shows different fractions had similar concentration.

Stage of gel filtration	Mean particle size in nm	Particle concentration in particles/mL
Fraction 1	163	9.9×10^8
Fraction 2	195	10.9×10^8
Fraction 3	103	9.2×10^8
Fraction 4	115	10.9×10^8
Fraction 5	176	10.6×10^8

Table 4.2: Liposome concentrations for the fractions from different stages of gel filtration as determined by NTA.

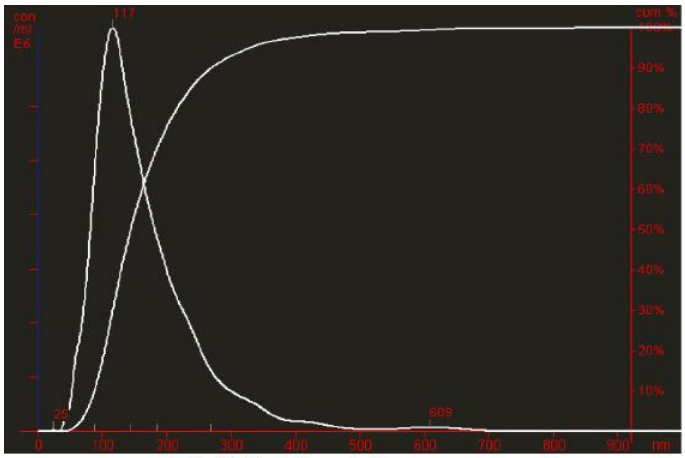


Figure 4.8: A typical curve of size distribution and particle concentration of liposome samples. On x-axis of the graph it has the size of the particle in nm and in y-axis it has the number of particles.

Latex sample with known diameter of 140 nm was analyzed as reference. For 140 nm latex sample, the obtained diameter was 133 nm with a standard deviation of 37 nm. Thus the analysis with the latex sample showed that the system worked accurately to determine the size of the reference sample. Five different set of data were obtained from the nanoparticle tracking analysis for the final liposome sample. From the average of these results, it was obtained that the mean diameter of the liposomes were 203 nm with a standard deviation of 52 nm.

Stability in different media

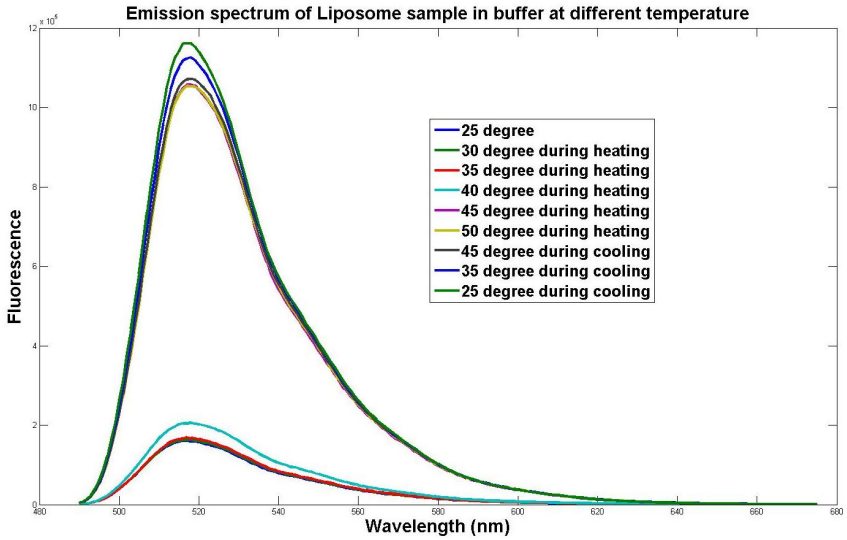
Liposome samples were analyzed to investigate the effect of the elevation of temperature. First the sample was heated with the heating system connected to the fluorometer and it is shown in figure 4.9 that the fluorescent signal increased significantly between 40°C-45° C, which signifies the release of CF from the liposomes. The fluorescence signal did not change with further increase of temperature and it also remained the same while cooling the sample. The liposome sample which was heated with the hyperthermia system also had a high fluorescence signal. The contrast of the color of the liposome samples before and after heated with the hyperthermia setup was clearly distinctive.

Liposome samples were examined in different media to investigate their stability. Fluorescence signal were high for the liposomes in glycerol and an equal volume mixture of PEG and PBS solution. After heating these samples, the fluorescence signal did not change. However, the liposome sample in PBS buffer was stable and showed similar fluorescence signals before and after heating for a time period of 10 days.

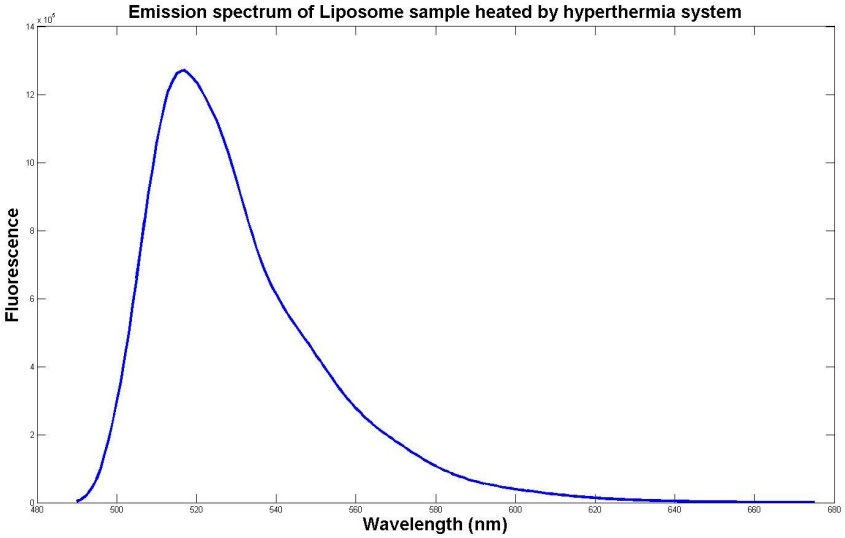
4.4 Demonstration of localized release from liposomes

Figure 4.11 is the thermal image of the muscle phantom, which depicted the hot spot where the hyperthermia system was focusing.

The liposome array was heated with the hyperthermia system for 25 minutes until the temperature readings showed 41°C. It was observed that the CF was released from all the points in the array at the bottom part of the columns. Out of 4 cm column of the liposomes, the release took place for the lower 2.5 cm and fluorescence could not be seen in the upper 1.5 cm part of the column.



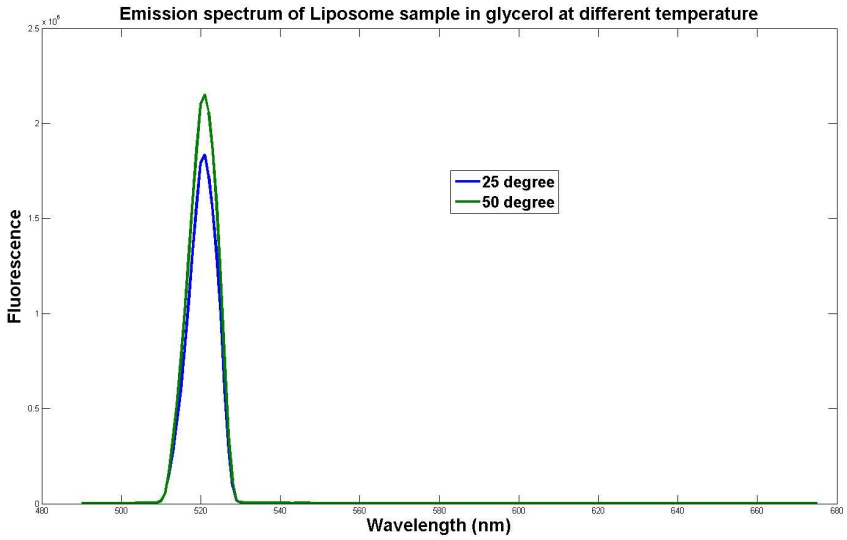
(a) Emission spectra of liposome heated by fluorometer system.



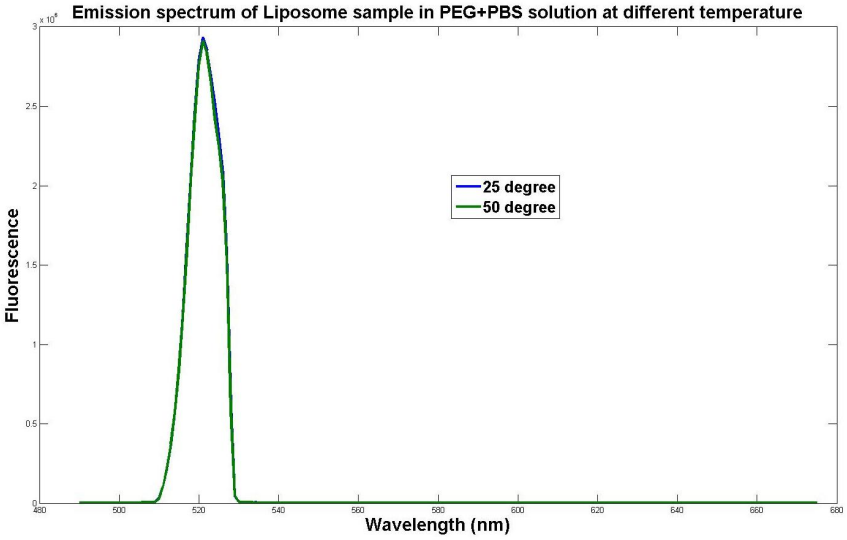
(b) Emission spectra of liposome heated by hyperthermia system.

(c) Liposome sample before and after heating with hyperthermia system.

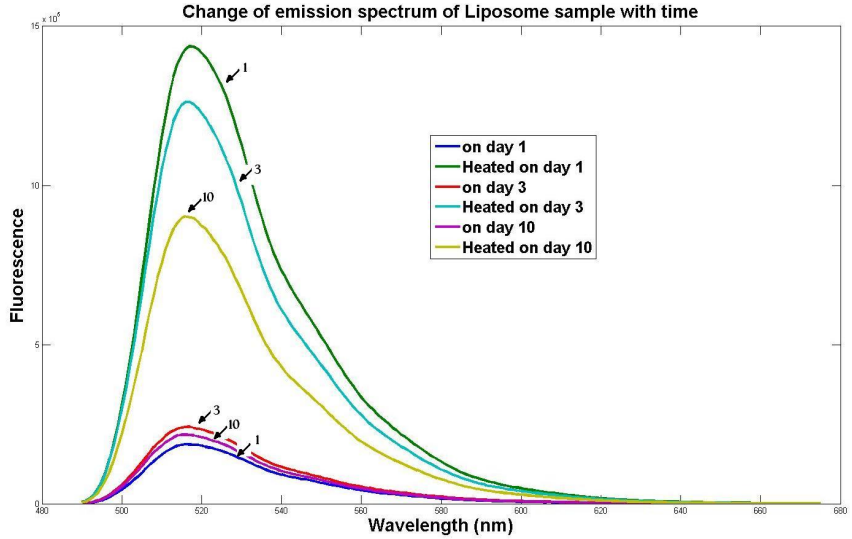
Figure 4.9: Fluorescence signal from liposomes.



(a) Emission spectra of liposome in glycerol.



(b) Emission spectra of liposome in PEG+PBS.



(c) Emission spectra of liposome in PBS with time.

Figure 4.10: Stability of liposome in different media.

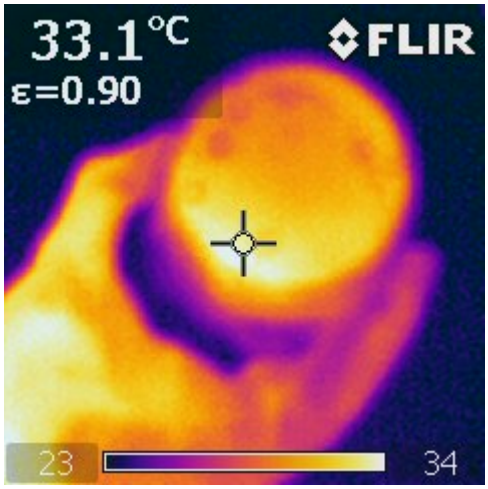
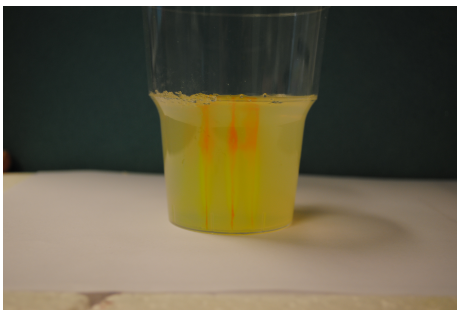


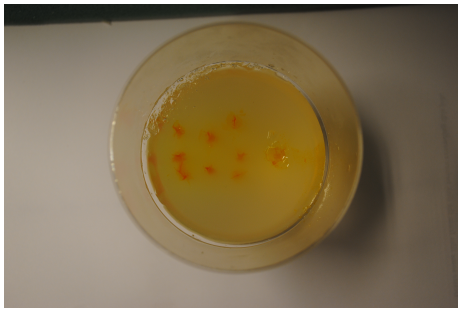
Figure 4.11: Thermal image of the muscle phantom.

Figure 4.12 demonstrates the localized release of CF from the liposome array induced

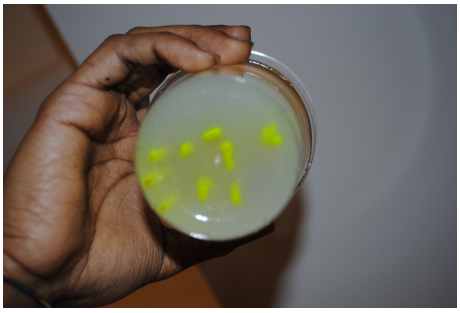
(a) Side-view of liposome array before heating.



(b) Side-view of liposome array after heating.



(c) Top-view of liposome array before heating.



(d) Bottom-view of liposome array after heating.

Figure 4.12: Phantoms with liposome array before and after heating with the hyperthermia system.

by the hyperthermia system.

5 Discussion

5.1 Characterization of liposomes

The results from the CF characterization experiments showed that CF was quenched for concentrations above 30 mM and was fluorescent for concentrations below 300 mM. This result complies with the assertion of Chen and Knutson as they described, CF has the property of self quenching at concentration higher than 200 mM [29].

The results obtained in the fluorometric analysis have shown that CF was released from the liposomes at a temperature between 40-45°C. The experiments with the hyperthermia set up showed that when the temperature reached to 41°C, the liposome solution became fluorescent. From these results, it can be concluded that CF released at 41°C. This result complies with the specification of the gel to liquide transition temperature for DPPC (41°C).

Nanoparticle tracking analysis was carried out for five consecutive fractions of liposome samples from different stages of gel filtration. It was predicted that the number of liposomes would gradually decrease for each additional fraction. But the results obtained from the NTA measurements showed the number of liposome in all the samples were almost in the same range in table 4.2. It was also difficult to obtain a smooth graph from the nanosight software as it is dependent on the operators skill both in terms of handling the sample, finding out right concentration for performing the measurement as well as manipulating the camera settings such as the brightness, blur, gain etc. During the experiment, the liposomes were observed to interact with each other and this might also have altered the results. Latex samples were used as reference sample to check the results. The auto-detection mode in the software worked well with the latex sample but for the liposomes, it did not produce smooth curves. The reason might be that the latex reference samples were more mono-disperse than the liposome sample. The polydispersity of the liposome sample might have altered the resulting size distribution as the settings for the auto-detection could not locate the smaller and the larger object simultaneously. The relative intensity of the liposome samples might be very high due to the polydispersity and it might have evoked the difficulty for the setup to detect the smaller and the larger particles simultaneously. However, other techniques such as Dynamic Light Scattering (DLS) can be applied to verify the results obtained by the nanoparticle tracking analysis.

5.2 Demonstration of local release of CF

It was expected that the fluorescence would be observed in only a certain portion of the liposome array. However, CF was released from all the spots of the array. In vertical direction, the release of carboxyfluorescein was obtained in the bottom part of the gel upto 2.5 cm from the bottom surface. For the upper 1.5 cm of the liposome columns, the fluorescence could not be observed. This suggests that the temperature did not reach to 41°C at this upper part of the gel. In a cross section it was expected that the focusing point should be near to the probe 1 (from figure 4.4). As the release could be found in all the spots in cross section, to investigate the reason, another muscle phantom was heated with the hyperthermia system for 25 minutes with the same settings and thermal images were taken as shown in 5.1.

The thermal image shows that the temperature at the focusing point reached more that 43°C after 25 minutes of heating and in the surrounding it is also high enough to induce the

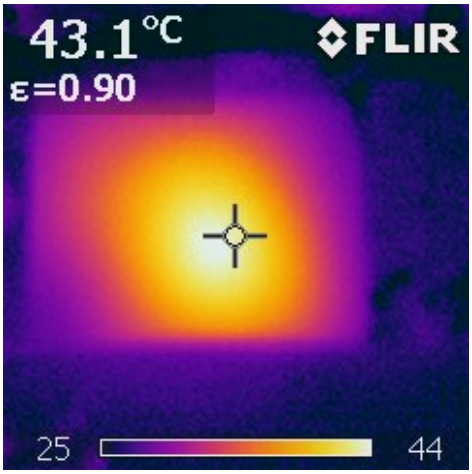


Figure 5.1: Thermal image of the muscle phantom for the trial experiment.

release of CF. This reveales that the phantom was heated for longer time which resulted in elevated temperature all over the bottom part of the Liposome phantom to induce CF release. Thus, the hyperthermia system was successful to induce the localized release of CF from the liposomes. However, the experiment needs to be optimized to improve the resolution of the localized heating.

5.3 Relevance for cancer drug delivery

Combination of regional hyperthermia and thermosensitive liposomes for bimodal treatment of cancer has the potential to meet the challenge of targeting the drug into the specific site of action. For mild hyperthermia treatment of cancer, the temperature is kept at 40-41°C at the tumor tissue, which makes the cell more susceptible to the effect of the drug [12]. The DPPC liposomes in this thesis project were able to release the content at 41°C, which can be an effective temperature for using with mild hyperthermia. Liposomes are biodegradable, biocompatible and they do not evoke immune response [28]. However, the challenge facing by the liposomal drug delivery is to release the encapsulated drug only at the specific site at controllable rate. Liposome themselves are not toxic to the cell, but toxicity of the incapsulated drug can be an issue. Dose of the drug can be planned by mimicking the array format used in this project to release the drug only at the site of action. In many applications it will be required to first accumulate the drug at its site of action (ie. before release) to reduce systemic effects of the drug.

6 Conclusion

In the first part of this project, DPPC liposomes with encapsulated CF were successfully prepared. They were shown to be stable in PBS over time and to release CF at 41° C (the T_m of DPPC). Once the CF was released from the liposomes, the fluorescent signal did not change with cooling. As expected, the maximum fluorescent signal for CF samples was obtained for concentrations ranging between 3 mM to 0.3 mM. The mean diameter of the liposomes was determined to around 200 nm with a standard deviation of around 50 nm.

In the second part of this project, a muscle phantom assembly was designed for the demonstration of local release of CF, where CF-containing liposomes were injected in an array to reduce material consumption and to mimic targeted drug delivery. Using this phantom, a hyperthermia system was successful to induce localized release of CF from the liposomes. It will be possible to enhance the resolution of the localized heating by optimizing the experiment.

As an extension of this work, CF could be used instead of drugs in more advanced model systems for further investigation of, for example, different approaches of targeted drug delivery. Furthermore, this model can be developed to use with animal models. It is a great challenge to bring together new ideas from the knowledge, experience and development of several technologies such as nanotechnology, polymer chemistry and electronics to develop drug delivery systems. Drug delivery systems for cancer treatment are no longer just encapsulating the drug inside a polymer for delivery through different routes. New approaches to cancer treatment not only supplement the conventional chemotherapic or radiotherapic treatments, but also aim to prevent damages to normal tissue and to reduce drug toxicity. Bimodal cancer treatments approach can play a vital role to provide solution to these challenges.

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