TOWARDS A MORE EFFICIENT ULTRASOUND RESPONSIVE DRUG DELIVERY SYSTEM:
A FEASIBILITY STUDY

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June 5th, 2012

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SUMMARY

To circumvent the undesired side-effects of certain drug delivery systems, a treatment method has to be developed that permits delivery of drugs only at a certain target site, for example chemotherapeutic drugs at a tumor site. An interesting approach could be the design of drug carriers which respond and release their content upon external stimuli. Ultrasound can be suggested as such a stimulus as it creates a directed external force to trigger drug release. The drug delivery system in this thesis is built of ultrasound responsive microbubbles to which Doxorubicin (DOX)-containing liposomes are attached as little pods. Two types of liposomes are created: a conventional type (Doxil) and a thermosensitive type. These last liposomes contain -next to the regular lipids- lysolipids, which lower the phase-transition from the solid to the liquid state when temperature is elevated and hence enhance drug release. Ultrasound exposure induces cavitation of the microbubbles, which causes sonoporation of cell membranes.

In a first temperature-based experiment we proved that we indeed were able to produce thermosensitive liposomes. Subsequently, we wanted to evaluate the efficiency of drug release between the two different liposomal formulations. For this purpose, we performed an experiment to measure DOX release out of the two types of microbubble-linked liposomes upon ultrasound exposure. Ultrasound indeed, showed a more efficient release of DOX out of the thermosensitive liposomes compared to their Doxil-loaded counterparts. Finally, we evaluated the therapeutic efficiency of these systems in vitro with cell viability assays on BLM melanoma cells. These cells were killed if DOX was released out of the liposomes. The thermosensitive liposomes showed to be more cytotoxic than the conventional liposomes, mainly when ultrasound was applied. This increased cytotoxicity can be explained by an enhanced release of DOX out of the thermosensitive liposomes and their higher in-se toxic properties.

Another point we tackled in this thesis was the conversion of nanoemulsions into image-detectable microbubbles driven by heating. The nanoemulsions were evaluated pre- and post-heating but the detected pre-heating particles were too big, which forced us to consider further optimization of the nanoemulsion preparation and evaluation.
**SAMENVATTING**

Om de ongewenste neveneffecten van bepaalde geneesmiddelafgifte systemen te omzeilen, is er een behandelingswijze nodig die enkel afgifte van geneesmiddelen toestaat specifiek ter hoogte van een bepaald doelwit, bijvoorbeeld chemotherapeutica ter hoogte van een tumor. Een interessante benadering zou hier kunnen gevonden worden in het ontwerpen van geneesmiddeldragers die reageren op externe stimuli en hierbij ook hun inhoud vrijstellen. Ultrageluid kan hier worden voorgesteld als zo een stimulans, aangezien het een gerichte externe kracht kan voorzien die geneesmiddelvrijstelling stimuleert. Het geneesmiddelafgifte systeem dat in deze thesis werd geëvalueerd is opgebouwd uit ultrageluid-responsieve microbellen waaraan Doxorubicine (DOX)-bevattende liposomen zijn gekoppeld. Twee types liposomen werden ontwikkeld: een conventioneel type (Doxil) en een warmtegevoelig type. Dit laatste type bevat naast de gebruikelijke lipiden ook nog lysolipiden, die de fase-transitie van de lipide membraan verlagen wanneer de omgevingstemperatuur verhoogd is, wat zou moeten leiden tot een verbeterde afgifte van geneesmiddelen. Wanneer microbellen worden blootgesteld aan ultrageluid zullen deze caviteren, wat uiteindelijk sonoporatie van celmembranen kan veroorzaken.

In een eerste experiment werd bewezen dat we inderdaad in staat waren om de warmtegevoelige liposomen aan te maken. Vervolgens werd de vrijstellingsefficiëntie van het geneesmiddel uit de twee verschillende microbellen-gekoppelde liposomale formuleringen geëvalueerd wanneer zij werden blootgesteld aan ultrageluid. Ultrageluid zorgde hier voor een efficiëntere afgifte van DOX uit de warmtegevoelige liposomen in vergelijking met de Doxil-geladen tegenhangers. Tenslotte werd ook de therapeutische efficiëntie van deze systemen in-vitro getest op BLM cellen. Deze cellen werden gedood als DOX werd vrijgesteld uit de liposomen. De warmtegevoelige liposomen bleken meer cytotoxisch dan de Doxil liposomen, vooral wanneer ultrageluid werd uitgeoefend. Deze verhoogde cytotoxiciteit kan worden verklaard door een verhoogde afgifte van DOX uit de warmtegevoelige liposomen en hogere in-se toxische eigenschappen van dit type liposomen.

Een ander punt dat werd aangehaald in deze thesis was de door warmte gedreven omzetting van nanoemulsies in detecteerbare microbellen. De nanoemulsies werden voor en na verwarming geëvalueerd, maar de deeltjes die voor verwarmen werden gemeten bleken te groot. Dit leidde tot de conclusie dat de nanoemulsiebereiding en -evaluatie moet herzien en geoptimaliseerd worden.
Deze thesis is het resultaat van mijn werk gedurende twaalf weken in het Laboratorium voor Algemene Chemie en Fysische Farmacie in Gent. Bij deze zou ik ten eerste Prof. dr. J. Demeester, Prof. dr. S. De Smedt en Prof. dr. K. Braeckmans willen bedanken voor de nodige faciliteiten die zij mij hebben aangeboden en voor de gelegenheid die zij mij hebben gegeven om in hun labo te mogen werken.

Een tweede “dank u wel” verdient mijn begeleider Bart Geers, voor de uitstekende begeleiding op elk vlak, de bereidheid om al mijn vragen uitgebreid te beantwoorden en zijn enthousiasme voor het vak waarmee hij mij de interesse voor het onderwerp aanwakkerde.

Hierop volgend wil ik ook de andere thesisstudenten bedanken - in het bijzonder Joke en Rein - voor de toffe sfeer die ik heb mogen ervaren in het labo tijdens deze onderzoeksstage en voor de ontspannende middagpauzes.

Hiernaast verdienen ook zeker mijn mama en papa hun plaatsje in deze tekst, want zij zijn immers diegenen die mij deze studiekans gegeven hebben en mij onvoorwaardelijk steunen.

Ook Ann, Veerle en Koen wil ik bedanken om hun tips en ervaringen te delen en in de weekends voor de nodige ontspanning te zorgen thuis. Als laatste een merci aan Jasper voor alle steun en “ontstressing” die hij mij de afgelopen maanden heeft gegeven.
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LIST OF USED ABBREVIATIONS

CO₂: Carbon Dioxide
DC: Duty Cycle
DLS: Dynamic Light Scattering
DMEM-F12: Dulbecco’s Modified Eagle Medium F-12
DMSO: Dimethylsulphoxide
DNA: Deoxyribose Nucleic Acid
DOX: Doxorubicin
DPBS: Dulbecco’s Phosphate buffered saline
DPPC: 1,2–dipalmitoyl–sn–glycero–3–phosphocholine
DSPE–PEG–PDP: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[PDP(polyethylene glycol)-2000]
EPR: Enhanced Permeability and Retention
FDA: Food and Drug Administration
GC: Gas chromatography
MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
(NH₄)₂SO₄: Ammonium sulfate
FBS: Fetal Bovine Serum
PEG: Polyethyleneglycol
PFP: Perfluoropentane
SPT: Single Particle Tracking
TRITON X: Triton X-100
TSL: Temperature Sensitive Liposome
US: Ultrasound
UV/VIS: Ultraviolet/Visible light
VEGF: Vascular Endothelial Growth Factor
1. INTRODUCTION

1.1 CANCER THERAPY

Cancer is a group of diseases characterized by the uncontrolled multiplication of cells. A group of these cells can form a local slug, named a tumor. The proliferating cells can spread out to neighboring tissues. In severe cases, they can reach the bloodstream via the lymphatic system and travel around in the body. They can urge other cells, belonging to healthy tissues and organs, to differentiate into tumor cells. This process -called metastasis- is the most severe form of cancer and can establish damage to the whole body. Cancers are caused by abnormalities in the genetic material of the transformed cells. Carcinogens, such as tobacco smoke, radiation, chemicals or infectious agents can be the source of these abnormalities.

The first line treatment for tumors is surgery. It is the only intervention needed for a lot of small tumors, however an early diagnosis is essential. Another effective treatment is radiotherapy. Nevertheless, the radiation can cause unpleasant damage, such as local alopecia or nausea. A third form of handling cancer is chemotherapy, which focuses on influencing the cell cycle. Normal cells only undergo multiplication upon signaling. Cancer cells do not react upon these signals and split continuously. Chemotherapy tries to stop the uncontrolled cell growth of the tumor by using anticancer drugs, e.g.: Taxol, Cispplatin and Doxorubicin. As chemotherapy affects cell division, tumors with high growth fractions are more sensitive to chemotherapy. But it brings problems with it as well. First, resistance to certain chemotherapeutic drugs can take place when long-term administration is needed. Another problem is the deficiency of specificity of drug delivery, which is due to the systemic administration. Hence, most of the chemotherapeutic drugs do not only operate at the tumor site, but also affect normal cells and this causes undesired side-effects.

To circumvent all these side-effects, a treatment method has to be developed that permits delivery of drugs only at a certain target site, for example a tumor. An interesting approach could be the design of drug carriers which respond and release their content upon external stimuli. Ultrasound can be suggested as such a stimulus as it creates a directed external force to trigger drug release. Hence, it leaves all the other tissues unharmed and site directed drug delivery can be achieved (see figure 1-1).
Figure 1-1: The side-effects of standard chemotherapy treatment of tumors compared to site-directed drug delivery (http://members.chello.nl/rwielens/).

1.2 ULTRASOUND

1.2.1 General aspects

Ultrasonic waves are a branch of acoustics that cannot be processed by the human ear. The highest frequencies that can be heard go up to 20 kHz (1). Frequencies above this border belong to the ultrasonic area, which has an upper limit of 800 kHz. The waves, whose existence depends on vibrations, travel through media. These media have elastic properties to sustain the vibrations (1). The waves can be described in terms of amplitude, frequency and wavelength. The amplitude expresses the height of the wave. Frequency, having Hertz as unity, is the total of all vibrations that pass through one point in the medium per second. The wavelength is measured by determining the distance between two maxima of neighboring waves.

The machinery used to produce ultrasound mainly consists of a generator and a transducer (figure 1-2). The generator sends out an alternating current to the transducer. The piezoelectric crystal, built into the transducer, will start to vibrate. Hereby, the electrical signal will be alternated into a pressure wave and soundwaves will be sent out. The transducer can also receive these soundwaves. Thus, when soundwaves hit the crystals, they will emit electrical pulses.
Ultrasound is a mechanical force that is proportional to pressure. The frequency is reversed-proportional to ultrasound: the higher the frequency, the smaller the mechanical force. Ultrasound creates pressure waves which vary from low to high pressure, as shown in figure 1-3. When soundwaves pass by an interface of different density, they can reflect and refract (2). Now, two waves exist: one that continues in the following medium and one that reflects and turns back into the initial medium (1). The reflections, known as “echoes”, are the basis for imaging in echography.

Figure 1-2: The building of a transducer. The transducer receives an electrical signal from the generator. The piezoelectric crystal starts to vibrate and sends out ultrasonic waves to the medium where it is applied to.

Figure 1-3: A soundwave in terms of pressure. The maximum of the waves is related to an increased pressure, the minimum to a decreased pressure.
1.2.2 Ultrasound as an imaging modality

1.2.2.1 General imaging properties of ultrasound

Ultrasound is widely used as clinical imaging tool for the body. Ultrasound is a non-invasive technique, it is cheap and it has a low risk to do serious harm (3). Unlike visible light waves, there is only little absorption of ultrasound by water or tissues. Therefore, ultrasound can be used as a diagnostic tool to look inside the body (2). The soundwaves, which are sent out to a specific part of the body, will either pass through tissue or can be reflected back to the piezoelectric crystal probe. As mentioned before, this probe can alter the ultrasonic waves into electrical pulses which in turn can be processed by a computer. All the information is blended together in an image on screen. An example is shown in figure 1-4.

![Figure 1-4: An echography image of a child during pregnancy](http://www.echopraktijkzuid.nl/images/termijnecho3.jpg).

Ultrasound can be an interesting tool to specify the exact location and size of tumors as well. The imaging occurs at low average intensities and high frequencies (2). Lastly, ultrasound will trigger temperature elevation where it is applied (4). When the ultrasound is focused onto a small surface, the power in this area becomes very large and overheating can occur (2).

1.2.2.2 Contrast enhanced diagnostics for ultrasound imaging

But the imaging function of ultrasound gets limited when non-reflective substances have to be imaged, e.g.: blood. A solvation for this problem can be found in the help of contrast-agents, which can optimize the quality of the image. Especially when tumors are situated in low-density tissue zones, contrast agents will be necessary. The contrast-agents used for tumor imaging are microbubbles. Microbubbles react different to ultrasound stimuli than human blood or tissues. They can be followed up to the smallest vessels (5). Microbubbles oscillate upon insonification and this results in strong scattering of ultrasound.
Hence, they appear bright on an ultrasound image (5-7). An example of imaging with the help of microbubbles is shown in figure 1-5 B.

![Figure 1-5: A = ultrasound imaging before microbubbles are added as contrast agents. B = ultrasound imaging after microbubbles are added as contrast agents (http://prostate.tju.edu).](image)

Nowadays, microbubbles and ultrasound are used for therapeutic interventions as well, where they can be applied as local drug delivery system. The basic principles and mechanisms of action of these bubbles as drug delivery vehicles will be further explored in the following chapters.

1.3 MICROBUBBLES

1.3.1 A closer look at the composition of microbubbles

Microbubbles consist of a stabilizing shell around a gas-filled, hydrophobic cavity (see figure 1-6). The first microbubbles were filled with air, but these “air bubbles” dissolved in less than a second, due to the fast diffusion of air out of the bubble. The solution for this problem is the use of highly inert gasses. For this purpose, perfluorocarbon and hexafluoride gasses are the perfect agents (3). These gasses have a high water permeation resistance: the exterior water is less able to enter the microbubble when it is filled with these inert gasses, as compared to air (8).
Yet free perfluorocarbon microbubbles survive less than a minute. The dissolving of these newly formed bubbles, without encapsulation, inside the body is a consequence of surface tension at the gas-liquid interface (5). When a long-lasting existence of the microbubbles is intended, the microbubble “membrane” must be solid. This introduces the formulation of an encapsulating shell. This shell will give either resistance to gas that wants to diffuse out of the core and gives a reduction of the surface tension as well (5).

The shell material will affect the elasticity of the microbubble: the more elastic properties the shell has, the more volume expansion and contraction of the bubble can take place (9). Different types of shells are available: lipids, polymers or proteins can be used to form the shell.

1.3.1.1 Protein shells

When protein shells are used, albumin is frequently applied as encapsulating protein. The shell consists of a monomolecular layer of native and denatured albumin. Different cysteine residues, within the albumin molecule, can form disulfide bonds which hold the shell together. These covalent bindings lead to a certain rigidity of albumin shells when ultrasonic insonification is observed (10). The commercially available product “Albunex” was the first encapsulated microbubble composition that was approved by the Food and Drug Administration (FDA) for in vivo use (11). Other proteins than albumin can be used to coat microbubbles as well, but thiol groups must be present to form the disulfide bridges.
1.3.1.2 Polymer shells

Polymer shelled microbubbles have a flexible coating of polymers around their gas core. Natural or synthetic polymers can be used to stabilize the bubble. The bulky structure of polymers leads to a higher destruction threshold (12). This process enhances resistance to compression or expansion of the microbubbles. Hence, the echogenicity of the microbubbles will be reduced (10).

1.3.1.3 Lipid shells

Lastly, lipid-coated microbubbles can be considered. This type of microbubbles is encapsulated with phospholipids, which form spontaneously an orientated monolayer at the gas-liquid interface. These liquid shells are cohesive due to hydrophobic interactions between the acyl chains of the lipids. This manages the solid-like character of the lipid-shell (10). Lipid-coated microbubbles have more elastic properties than their counterparts (13).

Because of the flexibility of certain shells, specific interactions with acoustic waves can take place. These interactions can lead to implosion of the microbubbles and sonoporation.

1.3.2 How microbubbles react upon ultrasound stimuli

As soon as the microbubbles are exposed to the ultrasound, the cavitation process starts. If a pressure wave passes through the medium, the microbubbles will respond to this change. They will shrink at high pressures and expand at low pressures. This process of microbubble oscillation is called cavitation. There are two forms of cavitation: stable and inertial cavitation. Stable cavitation occurs when the applied acoustical pressure is low: the microbubbles will expand and shrink around a certain diameter (14). Stable cavitation creates strong liquid fluid streams around the bubbles, the so-called microstreams (15). Microstreams can apply shear stress on cell membranes which may result in a transient opening of the cell membranes. At higher ultrasound intensities, the microbubbles grow rapidly during the rarefaction phase, until they collapse. This collapse is due to the difference between the high-pressure medium outside the bubble and the low pressure inside the expanded microbubble. This results in the fragmentation of the microbubbles into many smaller bubbles. This process is called inertial cavitation.

The destruction of bubbles causes formation of high-energy microstreams, named microjets. The microjets cause shear stress on the membrane of cells and increases its
permeability (16). Next to these two events, bubble collapse can create high velocity jet streams as well. These are causing a local increase in temperature. This local heating will influence the phospholipid bilayer of cell membranes, which becomes more fluidly and more permeable for the bubbles (17).

If one is able to associate the microbubbles with drugs, these drugs can be released at the location where ultrasound is applied and therapeutic efficacy can be changed according to the bio-effects that are discussed above. There are different possibilities to associate drugs with the microbubbles.

1.3.3 Strategies to associate drugs with microbubbles

A first strategy is incorporation of the drug in the microbubble shell, which is known as shell complexation (17). Here, the lipophilicity of the drug and the shell have to be considered. Furthermore, drugs can be incorporated in an inner oil layer (18). Lastly, drugs can be incorporated in nanoparticles which can be linked to the microbubble shell as pods with the help of a covalent binding (17;19;20). Hence, an increased amount of drug can be loaded onto the microbubble shell. Liposomes are a promising option to function here as nanoparticles and will be further considered below.

1.4 LIPOSOMES

1.4.1 Liposomes: an introduction

Liposomes consist out of a phospholipid bilayer and an inner hydrophilic cavity. An image is shown in figure 1-7. When different (types of) phospholipids -which are amphiphilic molecules- are put together in water, they start to form liposomes. The lipid bilayer creates a permeability barrier which protects the internal content. This internal content can be loaded with hydrophilic drugs. The liposomal structure can vary from multilamellar vesicles with diameters of several microns, to small unilamellar vesicles with diameters in the range of several nanometers (21). The multilamellar vesicles can be transformed into single bilayer structures by the help of different techniques (21-23). Now, nano-sized liposomes are created which can function as drug carriers. For biomedical applications, particles with a diameter of 100-200 nm are mainly applied. As mentioned in section 1.3.4, it is possible to couple the liposomes to microbubbles. Hence, an efficient drug delivery system is built (21-23).
Figure 1-7: The liposome structure with a phospholipid bilayer and an hydrophilic core. The phospholipids consist of a polar head group (blue dots) and a hydrophobic tail.

Liposomes can be functionalized as well. This can be achieved by introducing functionalized lipids in the liposomal bilayer in different ways.

A first group are stealth liposomes, where lipids are conjugated to polyethylene glycol (PEG). Hence, opsonisation and recognition by the immune system can be circumvented, which leads to long lasting presence in the blood and enhanced stability (24).

Secondly, targeted liposomes can be created. An example here can be given by binding antibodies on the lipids. Now, antigens can be detected on cell surfaces, which leads to a very specific binding of the liposomes. Problems here can be caused by the large size of the liposomes and possible recognition by the immune system (24).

A last group of specialized liposomes are the temperature sensitive liposomes, from now on referred to as thermosensitive liposomes. This type of liposomes contains lysolipids in their bilayer structure as well. Lysolipids are a type of phospholipids that convert from their solid, crystal structure to a more fluid structure around 39,0°C. This melting process creates pores in the bilayer structure of the liposomes. To allow an increased drug release it may be worth to try thermosensitive liposomes in combination with microbubbles and ultrasound.
1.4.2 Thermosensitive liposomes

Thermosensitive liposomes (TSL’s) are liposomal structures that permeabilize if temperature is elevated (25). As with conventional liposomes, TSL’s can be loaded as well with a certain amount of drugs. By increasing temperature, their content will hence be released: transient gaps are formed in the bilayer which lead to permeability (26). The exact temperature at which this occurs is called the phase transition temperature. At this point the crystal-like acyl–chains of the lipids start to melt and this temperature is determined by the composition of the phospholipid bilayer. If regular phospholipids are used solo to form the bilayer, it will convert from its crystalline phase to the liquid phase around 43,0°C - 45,0°C (25). By adding lysolipids to the membrane, the phase transition can be increased and the phase-transition temperature can be lowered (27). Therefore, temperatures around 39,0°C can already lead to efficient drug release if the thermosensitive liposomes are loaded with a certain amount of drugs (25). The time during which release occurs is enhanced as well: maximum release is reached in nearly a few seconds of heating (28) (29). These mild elevations of temperature needed for enhanced release of drugs can be delivered by exposure of the liposomes to ultrasound.

1.4.3 Ultrasound induced temperature elevation and drug delivery

As ultrasound can be a stimulus for site-directed drug delivery with microbubbles, temperature can be such a stimulus as well. Mild hyperthermia influences (39,0°C – 41,0°C) will modify blood flow in tissues, cell membrane permeability and alter drug release out of liposomes (30). Indeed, fast drug release is desired to resist wash–out that is caused by the blood stream. TSL’s can be coupled to microbubbles and exposed to ultrasound. By doing so, sonoporation takes place and the ultrasound application can locally elevate temperature as well. This will lead to efficient and fast drug release out of the thermosensitive liposomes at the intended treatment site. In contrast to radiotherapy, acoustic energy captured by healthy tissues dissipates fast. Therefore, no thermal damage can be caused there by accumulation of heat (31). This indicates a safe and non-invasive drug delivery method.

Before the hyperthermia by ultrasound is applied, no drug release or extravasation takes place. The TSL’s are stable at 37,0°C and hold their content. Until this point, no free drug is available to interact with body tissues when in vivo application occurs. When temperature is raised to 42,0°C, strong extravasation and drug release occurs.
When it is upgraded to 45,0°C, there is no further extravasation, but still an improved release of drugs is noticed (32;33).

When chemotherapeutic drugs are included in the liposomal cavity, another advantage for administrating drugs by this drug delivery system is exploited, named the Enhanced Permeability and Retention (EPR) effect.

1.4.4 Enhanced Permeability and Retention (EPR) effect

It is known that nano-sized liposomes can take profit of the EPR effect in tumors. Tumors are characterized by a high angiogenesis process. This high angiogenesis is declared by an up-regulation of genes which produce the Vascular Endothelial Growth Factor (VEGF) in tumors. VEGF is the key substance needed for tumor angiogenesis: VEGF is known for stimulating the growth of new blood vessels from nearby capillaries. Moreover, it allows tumors as well to access the primary nutrients they need to grow (34). VEGF will start up a cascade reaction as it binds to its receptors, VEGF receptor-1, VEGF receptor-2 and VEGF receptor-3 (34). These receptors are expressed on vascular endothelial cells. Normally, endothelial cells in newly formed vasculature undergo apoptosis in the absence of survival signals. But VEGF is able to introduce anti-apoptotic signals, which leads to viability of the immature endothelial cells. Hence, VEGF allows new blood vessels to mature. Besides this, it takes care of remodeling processes and provides survival stimuli as well (34;35).

But the physiology of these newly formed blood vessels differs from normal body cells. First, the smooth muscle layer is absent, which causes a defect regulation of the blood flow to the tumor. Another difference are the enlarged cell junctions, which make the tumor vasculature more leaky. Cut-off sizes of the gaps here can range between 380 nm and 780 nm (36). This means that particles like liposomes, with a diameter around 200 nm, are able to penetrate in the tumor via this mechanism, there were penetration into normal tissue is impossible. At last, defect lymphatic drainage is noticed as well. All these elements contribute to a higher blood flow to the tumor site and a prolonged retention of the extravasated particles in the tumor tissue.

Now, one can speak of an enhanced permeability and retention, better know as the EPR effect. The EPR effect causes accumulation of (thermosensitive)liposomes and macromolecules at the tumor site. Thus, high local drug concentrations can be reached (37).
If now ultrasound is exposed to this tumor tissue, specific triggered release of drugs out of liposomes can occur as previously discussed in chapter 1.4.3 (see figure 1-8 B).

Figure 1-8: A shows the leaky vasculature of a tumor compared to healthy tissue. B shows how TSL’s (big green dots) release their content at the tumor site when mild hyperthermia is applied.

1.5 CHALLENGES IN DESIGNING SPECIFIC DRUG DELIVERY SYSTEMS

In the previous chapters, the formulation of a specific drug delivery system was discussed which consist of liposomes linked to microbubbles and ultrasound as a stimulus to release the drug from this system. Nevertheless, a few problems are involved with these drug delivery vehicles as well.

1.5.1 Drawbacks of the use of microbubbles

The most problems that correspond with microbubbles are due to their size and their function as drug delivery vehicles. If the microbubbles are too large, they can evoke an obstruction of the blood flow in blood capillaries, certainly inside the lungs (38;39). Next to this, microbubbles should be stable when they circulate, have a long circulation time,
extravasate through the tumor vasculature and then accumulate in the targeted tissue. But first of all, microbubbles do not circulate longer than a few minutes due to their gaseous properties (3). Moreover, as considered in chapter 1.4.4, tumors exist of a leaky vasculature with pores of maximum 780 nm. This size-cutoff leads to a problematically entrance of the micron-sized bubbles into the tumor.

A possible answer for this problem can be found in the development of perfluorocarbon nanoemulsions that are able to extravasate at the tumor site. Once present in the tumor, the nanoemulsions will be converted to microbubbles for efficient drug delivery and imaging. Another positive aspect is the enhanced length of circulation time of these nanoemulsions.

1.5.2 Characteristics of phase-shift nanoemulsions for usage as drug carriers

The perfluorocarbon nanoemulsions consist of nanodroplets which are stabilized by a lipid monolayer as surfactant which contain perfluorocarbons inside. Compared with gas-filled microbubbles, perfluorocarbon emulsions consist of liquid perfluoropentane (PFP) inside the cavity. The average diameter of the nanoemulsion droplets is about 250 nm, thereby avoiding pulmonary entrapment (39). By adding heat to the nano-emulsions, the droplets are converted into gas-containing nanobubbles due to the vaporization of PFP inside the bubble. When different nanobubbles coalescent, bigger microbubbles are created.

Nanoemulsions are small enough to extravasate through the leaky vasculature of tumors and enter the tumor tissue. If now heat is applied, microbubbles can be created inside the tumor and the microbubbles’ inherent extravasation problem is solved. By using appropriate compounds, the microbubbles can have echogenic properties as well, making them ideal vectors for ultrasonic tumor imaging (36;40-42).
2. **OBJECTIVES**

The main objective of this thesis is to evaluate the efficiency of drug release out of two alike specific drug delivery systems: temperature-sensitive liposomes, which contain lysolipids, coupled to microbubbles and regular liposomes coupled to microbubbles. The two different types of liposomes are filled with Doxorubicine (DOX), a chemotherapeutic drug (see figure 2-1). Besides this, we want to evaluate efficiency upon loading of microbubbles with these two different liposomal formulations as well.

To evaluate these two main objectives, we will first perform experiments to study whether we could actually design thermosensitive liposomes. Secondly, we want to evaluate the release of Doxorubicin out of the drug delivery systems upon ultrasound stimuli. Finally, we will design an experimental setting to evaluate in vitro the therapeutic efficiency of the liposome-microbubble systems with cell viability assays on melanoma cells.

![Diagram](image.png)

**Figure 2-1:** A closer look to the composition of both ultrasound-responsive drug delivery systems. Temperature-sensitive liposomes possess lysolipids in their liposomal bilayer, whereas conventional liposomes only contain regular lipids.

Another point we would like to tackle in this thesis is the conversion of nanoemulsions into image-detectable microbubbles. The size of the nanoemulsions before the transition will be measured. The conversion itself will be induced by heating up the test samples and the post-heating size will be evaluated as well.
3. MATERIALS AND METHODS

3.1 MICROBUBBLES

3.1.1 The composition of microbubbles

DPPC or 1,2-dipalmitoyl-sn-glycero-3-phosphocholin (figure 3-1) with a molecular weight of 734 g/mole was purchased from Lipoid GMBH, Ludwigshafen, Germany. A stock solution of 20.0 mg/ml was made in chloroform (CHCl₃). Chloroform was purchased from Sigma-Aldrich, Bornem, Belgium.

![Figure 3-1: The molecular structure of DPPC.](image)

DSPE-PEG-PDP or 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[PDP(polyethylene glycol)-2000] was purchased from Avanti Polar Lipids (Alabaster, USA) with a molecular weight of 2,99.10³ g/mole. A stock solution of 10,0 mg/ml was prepared in chloroform. The molecular structure of DSPE-PEG-PDP is presented in figure 3-2.

![Figure 3-2: The molecular structure of DSPE-PEG-PDP.](image)

3.1.2 Other solutions and gasses

Hepes buffer or (N-(2-hydroxyethyl)piperazine-N’-(2-ethaansulfonzuur) is often used in biological studies. A stock solution of 50,0 mmole/L at pH 7,4 was made. Hepes powder was purchased from Sigma-Aldrich (Bornem, Belgium) and was solved in distillated water.

Glycerol 99,0% was purchased from Chem-Lab nv, Zedelgem, Belgium.
Propyleneglycol (1,2-Propanediol, >99.5%) was purchased from Carl Roth (Karlsruhe, Germany) with a molecular weight of 76.1 g/mole.

Perfluorobutane gas, with a molecular weight of 238 g/mole, was purchased from F2 Chemicals Ltd, Preston, Lancashire, UK.

### 3.1.3 Preparation and characterization of microbubbles

For the preparation of 10.0 mL of microbubbles, the different lipids were mixed in a round bottom flask. The volumes and concentrations that were used are shown in table 3.1. After solvent evaporation (Rotavapor R-200, Büchi, Switzerland), a lipid film was obtained and subsequently 2.00 ml of propyleneglycol was added to dissolve the lipids. Meanwhile, a solution of 7.00 ml Heps and 1.00 mL of glycerol was made and heated up at 57.0°C. The lipid mixture was heated up again and the warm Heps–glycerol blend was added after a few minutes. The final 10.0 mL of mixture was divided over gas chromatography (GC)-vials and perfluorobutane gas was added. This occurred by using 2 needles, one to put the perfluorobutane gas in, and one needle to let air out of the vial. Lastly, microbubbles were activated by shaking the mixture during 15.0 seconds using the 3M ESPE Capmix™ (3M ESPE AG Dental Products, Diegem, Belgium). A stable microbubble emulsion must been formed for further experiments.

To characterize the microbubbles’ size and concentration, the Electrical Sensing Zone method was used by the help of the Multisizer™ 4 COULTER COUNTER® manual apparatus (Beckman Coulter, Inc., Brea, USA). First, 20.0 µL of microbubbles was suspended in 10.0 mL of electrolyte solution. Subsequently, the machine measured pulse differences in the electrolyte suspensions for a period of time which allowed the computer to calculate certain properties of the suspension such as concentration or the volume of the suspended particles.

**Table 3.1**: The different volumes and concentration of the lipid components of microbubbles, required to make 10.0 mL of microbubble-mixture.

<table>
<thead>
<tr>
<th>name</th>
<th>Concentration (mg/mL)</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>20,0</td>
<td>139</td>
</tr>
<tr>
<td>DSPE-PEG-PDP</td>
<td>10,0</td>
<td>58,0</td>
</tr>
</tbody>
</table>
3.2 LIPOSOMES

3.2.1 The composition of conventional liposomes

DPPC or 1,2-dipalmitoyl-sn-glycero-3-phosphocholine: details see section 3.1.1.

DSPE-PEG-mal or 1,2-distearoyl-sn-glycero-3-phosphoethanol-amine-N-[maleimide(polyethylene glycol)2000 (figure 3-3) was purchased as ammonium salt from Avanti Polar Lipids, Inc. (Alabaster, USA). The molecular weight came to $2.94 \times 10^3$ g/mole and a stock solution of 10.0 mg/ml was prepared in chloroform.

![Figure 3-3: The molecular structure of DSPE-PEG-mal.](image)

Cholesterol, shown in figure 3-4, was purchased from Sigma-Aldrich (Bornem, Belgium) with a molecular weight of 387 g/mole. A stock solution of 20.0 mg/ml in chloroform was made.

![Figure 3-4: The molecular structure of cholesterol.](image)

3.2.2 The composition of thermosensitive liposomes

DPPC and DSPE–PEG–mal: see section 3.1.1 and 3.2.1.

MPPC or 1-palmitoyl–2-hydroxy–sn–glycero–3–phosphocholine (figure 3-5) was purchased from Avanti Polar Lipids, Inc. (Alabaster, USA) with a molecular weight of 706 g/mole. A stock solution with a concentration of 13.3 mg/mL was made in chloroform and ethanol.
3.2.3 Other solutions

Hepes buffer: see section 3.1.2.

An ammonium sulfate stock solution \([\text{NH}_4\text{SO}_4]\) of 250 mM was used as well. Ammonium sulfate powder was purchased from Sigma-Aldrich, Bornem, Belgium with a molecular weight of 132 g/mole and was solved in distilled water.

3.2.4 Preparation of conventional liposomes and thermosensitive liposomes

For the preparation of 3,00 mL of both types of liposomes, the calculated volumes of the lipids (total lipid concentration: 16,0 mg/mL) were mixed a round bottom flask in their respective quantities (see table 3.2). Subsequently, 3,00 mL of a 250 mM ammonium sulfate stock solution was added to the lipid film that remained on the surface of the flask. Next, the lipid film was solved at 60,0°C and upon vortexing. Further, the warm lipid mixture was pushed through a Nuclepore® 200 nm membrane (Whatman, Kent, UK) by using a liposome extruder (AVESTIN Europe GmbH, Mannheim, Germany). The liposomes were collected in a tube and 500 µL of these nano-sized liposomes was put together with 4,50 mL of Hepes buffer in a BECKMAN® centrifuge tube (Beckman Coulter, Inc., Brea, USA). The liposomes were ultracentrifugated (BECKMAN COULTER L8 -70M Ultracentrifuge, Beckman Coulter, Inc., Brea, USA) for one hour at 25,0°C and hence formed a pellet on the bottom of the centrifuge tube. Finally, the supernatant fluid was removed and the pellet was solved in 500 µL of Hepes buffer.
Table 3.2: The different volumes and concentration of the lipid components of liposomes, required to make 3,00 mL of liposomes (16,0 mg/mL).

<table>
<thead>
<tr>
<th>Liposome type</th>
<th>Lipid name</th>
<th>Concentration (mg/mL)</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional</td>
<td>DPPC</td>
<td>20,0</td>
<td>915</td>
</tr>
<tr>
<td></td>
<td>DSPE-PEG-mal</td>
<td>10,0</td>
<td>22,7*10^2</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
<td>20,0</td>
<td>350</td>
</tr>
<tr>
<td>Thermosensitive</td>
<td>DPPC</td>
<td>20,0</td>
<td>10,6*10^2</td>
</tr>
<tr>
<td></td>
<td>DSPE-PEG-mal</td>
<td>10,0</td>
<td>10,1*10^2</td>
</tr>
<tr>
<td></td>
<td>MPPC</td>
<td>13,3</td>
<td>50,2</td>
</tr>
</tbody>
</table>

3.3 PREPARATION AND CHARACTERIZATION OF DOX-LOADED LIPOSOMES

3.3.1 Doxorubicin

As stated in the introduction, liposomes can be loaded with chemotherapeutic drugs. Here, Doxorubicin (DOX) or Adriamycin appears to be an excellent anticancer drug to be stacked up inside the liposomes’ hydrophilic cavity. DOX, of which the molecular structure is shown in figure 3-6, is classified under the group of the anthracyclines, which have a well-known cytotoxic activity, varying from DNA intercalation and topoisomerase II inhibition to lipid peroxidation and formation of free radicals (43). By loading DOX inside the liposomes, it will be less detrimental while it circulates inside the body because no free drug is available (43;44). Doxorubicin hydrochloride (10,0 mg/mL), was delivered by Sigma-Aldrich (Bornem, Belgium) with a molecular weight of 580 g/mole.

![Figure 3-6: The molecular structure of Doxorubicin.](image)
3.3.2 Active loading of Doxorubicin into liposomes

Subsequent to liposome production, 50,0 µl of 10,0 mg/mL DOX solution was added to 450 µL of liposomal dispersion as prepared in section 3.2.4. Afterwards, liposomes were incubated for two hours in a heating device at 35,0°C or 50,0°C for thermosensitive DOX liposomes and conventional DOX liposomes respectively. The exact temperature regulation must be followed up because it arranges a certain liquidity of the liposomal bilayer, which allows DOX entering the core. But, DOX can only cross the double membrane if it is not protonated. At the moment it enters the core, ammonium sulfate -which was already present in the hydrophilic cavity- can protonate DOX. Thus, precipitation occurs and hence high amounts of DOX can be trapped inside the liposomes‘ cavity (see figure 3-7).

Upon incubation, the excess of DOX was removed by ultracentrifugation for one hour at 25,0°C. Only the DOX-containing liposomes, which had formed a pellet on the bottom of the centrifuge tube, were used for further experiments. Finally, the pellet was mixed with 500 µL of Hepes buffer. The liposomes were further named as “Doxil” (conventional type) and “Thermodox” (thermosensitive liposomes).

![Diagram of the loading process of DOX inside a liposome.](image)

Figure 3-7: The loading process of DOX inside a liposome.
3.3.3 Determining the concentration of DOX loaded inside the liposomes by the help of UV/VIS

3.3.3.1 UV/VIS: theoretical overview

With the Ultraviolet/Visible light (UV/VIS) method, light extinction or absorption by solutions can be measured. According to the law of Lambert-Beer (see formula 3.1), there is a linear correlation between the concentration of a solution and the absorption of light at certain wave length, by this solution. Next to these two components, the distance that is covered by light waves and the molar extinction coefficient contribute to the absorption as well.

UV/VIS spectroscopy sends out a monochromatic light beam, which belongs to either the UV or the VIS light range. This light enters the solution and a certain fraction of light is absorbed. The intensity of the light, which leaves the solution, is measured. This intensity is lower than the incident rays of light. By using the formulas 3.1 and 3.2 below, absorbance and concentration of the DOX liposomes can be calculated.

Another method to calculate the unknown concentration is delivered by setting up a calibration curve, which was used in our experiments. The absorbance of stock solutions with different concentrations was measured and the unidentified concentration was derived from the curve.

Law of Lambert-Beer:

\[ A = \varepsilon \cdot c \cdot d \]  \hspace{2cm} (3.1)

In which:  
- \( A \) = absorption  
- \( C \) = concentration (mole/L)  
- \( \varepsilon \) = molar extinction coefficient (L/mole/cm)  
- \( d \) = distance (cm)

\[ \frac{I_1}{I_0} = 10^{-A} \]  \hspace{2cm} (3.2)

In which:  
- \( I_1 \) = resigned intensity  
- \( I_0 \) = incident light  
- \( A \) = absorption
3.3.3.2 Determining the loading efficacy of DOX inside the liposomes

UV/VIS was used to determine the concentration of Doxorubicin inside the liposomes. Therefore, the detergent Triton X-100 (Triton X) (10.0% w/w, Sigma-Aldrich, Bornem, Belgium) was added, which dissolved the liposomes. Hence, the content of the liposomes was released and was feasible to measure.

To start, a dilution series of pure DOX in Hepes buffer was made, with concentrations varying from 1,00 mg/mL to 0.0625 mg/mL. Hereafter, 100 µL of each type of DOX-loaded liposomes was diluted in two different Eppendorf® cups with 80.0 µL of Hepes buffer and 20.0 µL of Triton X. Of all these mixtures, 50.0 µL was pipetted in a transparent 96-well plate in threefold. Subsequently, absorptions were measured at 480 nm by using the Wallac 2100 EnVision™ plate reader (Perkin Elmer, Waltham, USA). The dilutions series of DOX was the basis for the calibration curve and DOX-concentrations were calculated as described in section 3.3.3.1. Lastly, these concentrations were multiplied by two, according to the ½ dilution that was made.

3.4 COUPLING MICROBUBBLES WITH DRUG-LOADED LIPOSOMES

3.4.1 Theoretical overview

Mostly an avidin–biotin binding is used to link microbubbles to the nano-sized liposomes. Biotin can be included in both microbubbles and liposomes by incorporating the lipid DSPE-PEG-biotin into the liposomal bilayer. By adding the linker molecule avidin, a high–affinity binding with biotin can be established. But this binding is not suitable for in vivo applications due to the immunogenic nature of avidin. Hence, another covalent binding is used for in vivo applications, for example the formation of a thio-ether bridge. The microbubble monolayer contains, next to DPPC, a certain amount of DSPE-PEG-PDP, which possesses a disulfide binding. After adding a reductant, the disulfide binding is reduced to a thiol group. The newly formed thiol can interact with maleimide, that is part of DSPE-PEG-mal in the liposome structure and that possesses a double carbon binding. A stable thio-ether bridge can now be formed, which is shown in figure 3-8.
3.4.2 Practical aspects

Several GC-vials were filled with a certain amount of microbubbles mixture. A small amount of DL dithiothreitol reductant (Sigma-Aldrich, Bornem, Belgium) was put in the vials. Next, the liposomes and the gas were respectively added to the vials. In order to finish the process, the microbubble–liposome system was activated by shaking the mixture for 15.0 seconds using the 3M ESPE Capmix™.

3.5 CHARACTERIZATION OF DOX-RELEASE

3.5.1 Fluorescence: theoretical aspects

Certain molecules own fluorescent properties. The fluorescence process starts at the absorption of light of a certain wave length by the examined molecule. At this moment, electrons of the molecule can be excited. As the excitation process is finished, the electrons come back to their ground state, where energy can be released. This energy can be transmitted in the form of light. But this light possesses a lower amount of energy because part of the energy is lost as heat. Thus, the wave length of the light that is emitted is now longer than the wave length of the excitation light. By irradiating a solution with a monochromatic light beam, which has the optimal wave length, molecules can be transferred to their excitation state. As they go back to the ground state, the emitted light can be registered by a detector.

3.5.2 A general overview of measuring DOX release by fluorescence

Doxorubicin is know for its fluorescent properties. At the moment the liposomes are loaded with DOX, quenching occurs due to the high concentrations of DOX together. At this point only little fluorescence can be detected. As DOX is released, the emitted fluorescent light is measured. The release of DOX takes place, as mentioned before, by adding a
detergent which solves the liposomes, e.g.: Triton X. Besides detergent agents, temperature elevation or ultrasound exposure can be influences as well for DOX release out of liposomes.

3.5.2.1 Evaluation of temperature-triggered release of DOX

To evaluate temperature-triggered release of DOX out of the two types of liposomes, different samples were made. First of all, the different volumes of liposomes, Hepes and Triton X (1%) which are shown in table 3.3, were mixed in Eppendorf® cups in twofold. A positive and negative control were included as well. Next, the test samples were incubated for 30 minutes: test cup 1 at 37,0°C and cup 2 at 43,0°C. Afterwards, 50,0 µL of each sample was pipetted in threefold in a black fluorescence 96-well plate. Subsequently, the fluorescence values were measured using the Wallac 2100 EnVision™ plate reader. Finally, the percentage DOX release was calculated by using formula 3.3.

\[
\text{% DOX-release} = \frac{\text{fluorescence sample} - \text{fluorescence negative control}}{\text{fluorescence positive control} - \text{fluorescence negative control}}
\]  

(3.3)

Table 3.3: The different volumes needed of liposome mix, HEPES and Triton X to create the test samples.

<table>
<thead>
<tr>
<th>Type of liposome</th>
<th>Sample type</th>
<th>Volume liposome - mix (µL)</th>
<th>Volume of HEPES buffer (µL)</th>
<th>Volume of 1% Triton-X (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOXIL</td>
<td>Negative control</td>
<td>100</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Positive control</td>
<td>100</td>
<td>80,0</td>
<td>20,0</td>
</tr>
<tr>
<td></td>
<td>Test control</td>
<td>100</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>THERMODOX</td>
<td>Negative control</td>
<td>100</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Positive control</td>
<td>100</td>
<td>80,0</td>
<td>20,0</td>
</tr>
<tr>
<td></td>
<td>Test control</td>
<td>100</td>
<td>100</td>
<td>-</td>
</tr>
</tbody>
</table>

3.5.2.2 Evaluation of ultrasound-triggered release of DOX

For this experiment and further in vitro experiments, a certain ultrasound transparent surface had to be available if we wanted to apply ultrasound on a fluid or on cultured cells.
Opticells® (Nunc. Thermo Fisher Scientific, Langenselbold, Germany) were an ideal tool for this purpose because they provided this specific kind of surface. Opticells® are built of two parallel, gas-permeable and ultrasound transparent polystyrene membranes. Test fluids or cells were injected in between the two membranes by the help of a needle (see figure 3-9). If one worked with cells, the position of the Opticell® and gravity force allowed cells to spread out over one membrane.

![Injection of a test solution](http://stemcelllab.tripod.com/labEquip.htm)

**Figure 3-9:** The injection of a test solution in between the two parallel transparent polystyrene membranes of the Opticell® by the help of a needle.

Different test-settings were created for measuring US-triggered release of DOX out of the different types of liposomes. First, microbubble–liposome systems were prepared as described in section 3.4.2. Next, six GC vials which contained 900 µL microbubble mix and 100 µL regular liposomes and six GC vials which contained 900 µL microbubble mix and 100 µL thermosensitive liposomes were made (table 3.4).

Later, 12 Opticells® were marked and filled with 1,00 mL of the corresponding blends and 9,00 mL of Dulbecco’s Phosphate Buffered Saline (DPBS). 3 Opticells® of each type were exposed to ultrasound. Ultrasound was applied with the help of the Sonitron sonoporator (Richmar Artison sonitron 2000, Artison Corp., Inola, USA) for 30,0 seconds, at 50,0% Duty Cycle (DC) and 2,00 Watt/cm². The Opticells® were plunged in a basin of water with a rubber layer on the bottom, which absorbed the ultrasound waves. Lastly, the 12 Opticells® were drained and the fluorescent properties of the test samples were measured using the Wallac 2100 EnVision™ plate reader. To calculate the percentage DOX that was released, formula 3.4 was used. Here, the quotient was made between the liposomes plus ultrasound and the liposomes without ultrasound. This quotient was distracted with one and multiplied by 100.

\[
\% \text{DOX-release} = \left( \frac{\text{fluorescence sample treated with ultrasound}}{\text{fluorescence sample treated without ultrasound}} - 1 \right) \times 100 \quad (3.4)
\]

Table 3.4: The different compositions of the GC-vials.

<table>
<thead>
<tr>
<th>Type of Liposome</th>
<th># GC vials</th>
<th>Volume of microbubble mix (µL)</th>
<th>Volume of liposomes (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxil</td>
<td>6</td>
<td>900</td>
<td>100</td>
</tr>
<tr>
<td>Thermodox</td>
<td>6</td>
<td>900</td>
<td>100</td>
</tr>
</tbody>
</table>

3.5.3 Evaluation of ideal fluorescence and concentration parameters

To evaluate fluorescence in relation to concentration, a dilution series of DOX-containing liposomes was made which varied from a 1-in-2 to a 1-in-32 dilution. The same dilutions were made for liposomes to which 10,0% Triton X was added as well. Next, 50,0 µL of these test solutions was pipetted in a black 96-well plate and to finish the test and fluorescence was measured of all test samples.

3.6 IN VITRO ASSAYS

3.6.1 Cell culture

BLM melanoma cells were used in all the in vitro tests. The cells were cultured at 37,0°C and at a 5,00% Carbon Dioxide (CO₂) atmosphere in an incubator. The cells were grown in polystyrene culture flasks in the presence of liquid medium which consisted of essential growth components, shown in table 3.5.

Table 3.5: The components and their volumes needed to make 500 mL of BLM medium.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s modified eagle medium (DMEM F12) (Gibco by Lifetechnologies, Merelbeke, Belgium)</td>
<td>425</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS)</td>
<td>50,0</td>
</tr>
<tr>
<td>Hepes buffer (1,00 M)</td>
<td>10,0</td>
</tr>
<tr>
<td>Penicilline / Streptomycin (100 IU/mL / 100 µL/ml)</td>
<td>10,0</td>
</tr>
<tr>
<td>L-Glutamine (2,00 mM)</td>
<td>5,00</td>
</tr>
</tbody>
</table>

To keep a good cell viability balance, the BLM cells were split up two times a week. To start, the medium was removed and the cells were washed with 10,0 mL of DPBS. Subsequently, 6,00 mL of trypsin (0,0500%) was added at a 20,0 mL polystyrene culture flask.
to release the cells. After a few minutes, trypsinisation was inactivated by adding 14,0 mL of medium. Following, two times 5,00 mL of this suspension was headed over to two new polystyrene culture flasks and 15,0 mL of medium was added in each flask. These flasks were put in the incubator until a cell-confluence level of 80,0% was reached. The confluent cells were plated in Opticells® (see section 3.5.2.2), but before this occurred, the concentration of cultured cells was calculated. Therefore, 2 culture flasks received each 3,00 mL of 0,0500% trypsin to release the cells from the bottom. Next, 7,00 mL medium was used for inactivating the trypsinisation process and 50,0 µL of these cell suspensions was put in an Eppendorf® cup followed by adding 100 µL of Trypan Blue (Sigma Aldrich, Bornem, Belgium). A droplet of this new mixture was put in a Bürker counting chamber. The cells were counted underneath the microscope, where they were seen as white dots on a blue background. In 3 big squares of each 16 little squares the cells were counted and the average was calculated. The cell concentration was calculated by the help of formula 3.5 below.

\[
\text{Cell concentration (cells/mL)} = \# \text{ counted cells} \times 10000 \times 3
\]  

(3.5)

If the concentration was calculated correctly, one computed the volume of cell suspension needed to fill one Opticell® with 1,00*10^6 cells (see formula 3.6).

\[
\text{Volume cell suspension per Opticell® (mL)} = \frac{1,00\times10^6(\text{cells per Opticell®})}{\text{cell concentration (cells per mL)}}
\]  

(3.6)

This volume was multiplied by the amount of Opticells® that had to be filled and was diluted with medium to obtain 10,0 mL fluid per Opticell®. As a final point, 20 Opticells® were filled and incubated for 48 hours at 37,0°C and 5,00% CO₂.

### 3.6.2 Ultrasound exposure to Opticells®

As cited in chapter 3.7.3, the cells were divided over Opticells®. After the incubation period, the cells were confluent enough to start the in vitro experiments. Before starting the tests, microbubble–liposome systems of 1,00 mL were developed which contained 100 µg/mL liposomes. The bubblemix itself was ½ diluted with Hepes. All vials were prepared with the same DOX-concentration.

To ensure comparable results, different test conditions were set up: a positive and negative control were included, some samples were irradiated with US, different types of liposomes were used, etc. All these different Opticell® variations are listed in table 3.6.
Subsequently, the Opticells© were taken out of the incubator, the cells were washed with 5.00 mL of DPBS and 9.70 mL (or 9.00 mL in case of the negative control) of medium was added to every Opticell©. Then, 300 µL test solution was added to the Opticells© and several samples were exposed to ultrasound for 15.0 seconds (2.00 Watt/cm²) (see table 3.7 for which Opticells© were irradiated with ultrasound). It is important to know that the US was applied on the side where the cells were attached and after the Opticell© was plunged into water. Finally, the Opticells© were labeled and were put back in the incubator. Subsequently, medium was taken out of the Opticells©, again a wash-step with DPBS took place, every Opticell© received 10.0 mL of fresh BLM medium and incubation took place for one night.

**Table 3.6: Different conditions and test solutions that were put in the Opticells©, which already contained 9.00 or 9.70 mL of BLM medium.**

<table>
<thead>
<tr>
<th>Sample name</th>
<th># Opticells©</th>
<th>Test solution</th>
<th>Ultrasound application (Yes/No)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxil + US</td>
<td>3</td>
<td>Microbubble – DOXIL mix 300 µL</td>
<td>YES</td>
</tr>
<tr>
<td>Doxil - US</td>
<td>3</td>
<td>Microbubble – DOXIL mix 300 µL</td>
<td>NO</td>
</tr>
<tr>
<td>Thermodox + US</td>
<td>3</td>
<td>Microbubble – THERMODOX mix 300 µL</td>
<td>YES</td>
</tr>
<tr>
<td>Thermodox - US</td>
<td>3</td>
<td>Microbubble – THERMODOX mix 300 µL</td>
<td>NO</td>
</tr>
<tr>
<td>Microbubbles</td>
<td>3</td>
<td>Microbubbles only 300 µL</td>
<td>YES</td>
</tr>
<tr>
<td>Negative control</td>
<td>2</td>
<td>Dimethylsulphoxid (DMSO) 1 mL</td>
<td>NO</td>
</tr>
<tr>
<td>Positive control</td>
<td>3</td>
<td>-</td>
<td>NO</td>
</tr>
</tbody>
</table>
3.6.3 MTT-assay

To test the cytotoxicity of the different test solutions, the MTT-assay was applied. This assay was executed by the help of the Cell Proliferation Kit I (Roche, Mannheim, Germany). To start, the present medium was removed out of the Opticells, they were washed with 5,00 mL DPBS and 0,500 mL of MTT-reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to every Opticell, which was completed with 4,50 mL BLM medium.

NAD(P)H, which is present in metabolic active cells and is related to mitochondrial activity, will induce ring opening within the yellow MTT-reagent to form the purple-colored formazan salt crystals. The reaction is shown in figure 3-10.

After a four-hour incubation period at 37,0°C, a second reagent was added to the cells which solved the formazan salt crystals. This process led to release of the purple color out of the cells after a night in the incubator. The more formazan detected, the more viable cells were present. Finally, the formazan was calorimetrically detected by using the Wallac 2100 EnVision™ plate reader. Absorbance levels were measured at 590 nm and the percentage of cell viability was calculated by formula 3.7.

\[
\% \text{ cell viability} = \frac{\text{absorbance sample at 590 nm} - \text{absorbance negative control at 590 nm}}{\text{absorbance positive control at 590 nm} - \text{absorbance negative control at 590 nm}}
\] (3.7)

Figure 3-10: MTT is transferred to formazan crystals by the help of the enzyme mitochondrial reductase, which is present in metabolic active cells.
3.6.4 Testing the intrinsic toxicity of liposomes upon cultured cells

To test the intrinsic toxicity of the liposomes on the BLM melanoma cells, an experiment in a 12-well plate was developed. To start, 1,00 mL of cell suspension was added to every well and the plate was incubated at 37,0°C for one day. The amount of cells in this one milliliter contained between 100*10^3 and 150*10^3 cells. Subsequently, the cells were subjected to the different treatments: 3 wells received each 10,0 µg DOX and 45 minutes incubation time and 3 wells received each 10,0 µg DOX and four hours incubation time. The same treatments were repeated for the other six wells, but here 5,00 µg of DOX was added to every well. Positive and negative controls with 100% survival of the cells and 0,00% cell viability (DMSO) were included as well. Afterwards, the cells were washed with DPBS, incubated and the cell viability was measured in every well by the help of the MTT-assay (see section 3.6.3).

3.7 NANOEMULSIONS

3.7.1 The lipid composition of nanoemulsions

DPPC and DSPE–PEG–mal: see section 3.1.1 and 3.2.1.

Cholesterol: see section 3.2.1

3.7.2 Other solutions

Hepes buffer: see section 3.1.2

Fluorescent label DiD oil was purchased from Invitrogen molecular probes (Merelbeke, Belgium).

Perfluoropentane 99,0% was purchased from Exfluor Research Corporation, RoundRock, TX, USA.

Flutec PP1 was purchased from F2 Chemicals Ltd, Preston, Lancashire, UK.

3.7.3 Preparation and characterization of nanoemulsions

For the preparation of 2,00 mL of nanoemulsion, the calculated volumes of the lipids (total lipid concentration: 16,0 mg/mL) and 10,0 µL of fluorescent label were mixed in a round bottom flask in their respective quantities (see table 3.7). After solvent evaporation (Rotavapor) 2,00 mL of Hepes buffer was added to the lipid film that remained on the surface of the flask. Subsequently, the lipid film was solved at 60,0°C and upon vortexing.
Two times 800 µL of this mixture was transferred into respectively two falcontubes and 100 µL of perfluoropentane and 100 µL of Flutec PP1 was pipetted underneath each solution to prevent vaporization. Hence, a two-phase system was formed which was emulsified by moving the tubes up and down alongside an activated sonication probe (probe model 102C CE, Branson Ultrasonics, Danbury, USA) Finally, the nanoemulsion mixture was extruded through a Nuclepore® 200 nm membrane.

Table 3.7: The different volumes and concentration needed to prepare 2,00 ml of nanoemulsion.

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration (mg/mL)</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>20,0</td>
<td>609</td>
</tr>
<tr>
<td>DSPE-PEG-mal</td>
<td>10,0</td>
<td>151*10^1</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>20,0</td>
<td>233</td>
</tr>
</tbody>
</table>

Referring to section 1.5.2 and 2, the aim of this test was to create a system in which conversion from nanodroplets into microbubbles could be observed. To detect this transformation, one measured the size of the particles before and after the phase-shift heating. Before heating, the particles were characterized by Dynamic Light Scattering (DLS) and Single Particle Tracking (SPT). Pre- and post-heating processes were evaluated with the Electrical Sensing Zone method (Coulter-Counter, Beckman Coulter, Brea, CA, USA). Before every characterization, 50,0 µL of the nanoemulsions was diluted in 1,00 mL Hepes buffer, which will be now referred to as “nanoemulsion dilution”.

3.7.3.1 Dynamic Light Scattering

The Dynamic Light Scattering (DLS) technique measures the velocity of Brownian motion of particles, which is defined by the diffusion coefficient, and relates this to the size of particles.

At the moment the sample was irradiated with laser light, we measured the rate at which the intensity of the scattered light fluctuated when it was detected. The rate at which these intensities fluctuations occurred depended on the size of the particles: the smaller the particles, the more Brownian motion and the more light scattering was detected. By knowing the viscosity and temperature of the medium, one could trace back the hydrodynamic diameter of a particle. The samples were prepared by mixing 20,0 µL of the nanoemulsion dilution in 50,0 µL of Hepes buffer.
### 3.7.3.2 Single Particle Tracking

Single Particle Tracking (SPT) is a microscopic analysis method which captures the motion of single particles in a film by making use of the fluorescent properties of the particles and a widefield microscope.

The different frames obtained by the EMCDD camera coupled to the widefield fluorescent microscope, were processed by means of image processing. The trajectories of the different particles could be calculated and this enabled us to calculate the mean square displacement of the different particles. The diffusion coefficient and the corresponding size could hence be calculated via the Stokes-Einstein equation (see formula 3.8).

Stokes-Einstein equation:

\[
D = \frac{k_B T}{3\pi \eta d_H} \quad (3.8)
\]

In which:
- \(D\) = diffusion coefficient \((m^2/s)\)
- \(k_B\) = Boltzmann’s constant \((J/K)\)
- \(T\) = absolute temperature \((K)\)
- \(\eta\) = viscosity \((kg/(m*s))\)
- \(d_H\) = hydrodynamic diameter of the spherical particle \((m)\)

### 3.7.3.3 Electrical Sensing Zone method

For this test, 20,0 \(\mu\)L of nanoemulsion dilution was suspended in 10,0 mL of DPBS. Other details were already discussed in section 3.1.3.

### 3.8 STATISTICAL ANALYSIS

In all tests, two samples were compared by the use of a two-sided unpaired Student’s t-test. Differences between the samples were considered statistically significant when the p-value was less than 0.05.
4. RESULTS

4.1 LIPOSOMES AND MICROBUBBLES

4.1.1 Evaluation of DOX concentration

![Figure 4-1: The Doxorubicin calibration curve. The curve is built up by plotting the different dilution concentrations (mg/mL) against their UV/VIS absorbance.](image)

The DOX concentration inside the liposomes was determined as described in section 3.3.3.2. The different dilution concentrations of the DOX stock solution, required to built up the calibration curve, were plotted against their UV/VIS absorbance values. A linear trend line and the calibration curve’s equation were added to the data set. The calibration curve is shown in figure 4-1. With the help of the equitation $Y=0.889x+0.0489$ the DOX concentration of every liposome stock was calculated.

4.1.2 Evaluation of ideal fluorescence and concentration parameters

To evaluate fluorescence in relation to concentration, a dilution series was set up as noted in chapter 3.5.3. One defined the fluorescence values of the liposomes with Triton X as 100% fluorescence, because all DOX was released under these circumstances. The data are shown in figure 4-2. More fluorescence is seen at higher concentrations. One notices an increase in the difference between the 100% curve and the curve without Triton-X when concentrations are higher.
Figure 4-2: The concentration of the different liposome types, with or without Triton X plotted against their fluorescence.

4.1.3 Evaluation of temperature–induced DOX release

A difference in DOX release between both types of liposomes when temperature is changed was tested at two different temperature-settings: 37,0°C and 43,0°C. The test was executed as described in section 3.5.2.1. The release at 37,0°C was used as negative control, because at this point, there was no release of DOX out of both types of liposomes. The positive control referred to full release of DOX out of the liposomes by Triton X. With the help of formula 3.3, the percentage of DOX release was calculated and is plotted in figure 4-3. The release out of the thermosensitive type was 63,0%, whereas the release of DOX out of the conventional liposomes showed a decrease of -13,6% counts at hyperthermia conditions. Thus, one notices a significant difference in release at hyperthermia conditions between the Doxil and the Thermodox formulation and this is confirmed by the p-value which was calculated at 0,0115.

Figure 4-3: The different test settings plotted against the percentage DOX release out of the two types of liposomes.
4.1.4 Evaluation of ultrasound-induced DOX release

The ultrasound-induced DOX release was determined by the protocol described in section 3.5.2.2. The percentages release were calculated as shown in formula 3.4 and are plotted in figure 4-4. The release of DOX by conventional liposomes measures 5,06%, whereas the release of DOX by the thermosensitive type of liposomes is calculated as 25,0%. This significant difference in release between the Doxil and the Thermodox formulation with ultrasound exposure is confirmed by the p-values listed in table 4.1.

Figure 4-4: The percentage DOX release plotted against the two liposomal formulations.

Table 4.1: Statistical analysis of the different test samples executed with a Student’s t-test.

<table>
<thead>
<tr>
<th>Sample</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxil + US ↔ Doxil - US</td>
<td>0,00295</td>
</tr>
<tr>
<td>Thermodox + US ↔ Thermodox - US</td>
<td>3,46\times10^{-10}</td>
</tr>
<tr>
<td>Doxil + US ↔ Thermodox + US</td>
<td>5,91\times10^{-10}</td>
</tr>
</tbody>
</table>

4.1.5 In vitro evaluation on melanoma cells

Different test samples were prepared and different test conditions were set up to test the toxicity on BLM melanoma cells as cited in section 3.6.2. The toxicity was determined by making use of the MTT-assay, which was explained in chapter 3.6.3 and the cell viability was calculated as in formula 3.7. All results were statistically evaluated and the respective p-values of the different tests are listed in table 4.2.

The first test was executed with an incubation time of four hours after every treatment (the different treatment methods are shown in table 3.6). A concentration of 100 µg liposomes per Opticell© was used and a 25,0% DC was applied if ultrasound was necessary.
The percentages cell viability are plotted in figure 4-5 A in dark grey. First of all, a large cell toxicity is mainly seen for all liposomal formulations, with or without ultrasound. The cell viability percentages vary from 7,59% (no ultrasound) to 3,08% (with ultrasound) for Doxil and a significant difference is seen for Thermodox liposomes which varies from 4,29% (no ultrasound) to 0,00% (with ultrasound). In both cases, the Thermodox formulation is more toxic for the cells than when Doxil was applied. Microbubbles and ultrasound exposure leads to 79,6% cell viability.

In the following test, the Duty Cycle was decreased to 20,0% while other parameters were preserved. Again, cell viability percentages were calculated and the results of the test are shown in figure 4-5 A in light grey. The cell viability of the Doxil type is elevated compared to the previous test, to 45,1% (without ultrasound) and 21,5% (with ultrasound). Thermodox with ultrasound stays at 0,00% cell viability, but the Thermodox test without ultrasound treatment shows less cytotoxic effects (17,6% cell viability), compared to the previous test with 25,0% DC. The cell viability percentage of microbubbles with ultrasound shows a decrease to 48,9 %.

In order to lower obtained cytotoxicity, only 30,0 µg of liposomes was added per Opticell®. A one-hour incubation period and a 20,0% DC were used as well in this third experiment. The percentages of cell viability are plotted against the different samples in figure 4-5 B in dark grey. The significant difference between samples with ultrasound and the samples without ultrasound treatment is again confirmed. Increased efficiency is seen when the Thermodox formulation was used: a decrease in cell viability of more than 50,0% is seen (from 55,6% to 1,48%). There where in case Doxil was used only a 15,0% reduction in cell viability is observed.

Finally, we lowered the used DC to 10,0% while keeping the other experimental conditions unvaried with regard to the previous experiment (30,0 µg DOX used and one hour incubation time). The results are shown in figure 4-5 B in light grey. First, one can clearly observe a reduction in cytotoxicity upon ultrasound application of 88,0% in case Thermodox was loaded on the bubbles’ surface (from 92,9% to 4,19%; p = 6,72*10^{-15}). The Doxil efficiency varies from 71,1% to 28,5%, which leads to an efficiency of more than 40,0% (p=1,74*10^{-9}). The microbubbles and ultrasound resulted in 84,8% cell viability.
Figure 4-5: The percentages cell viability plotted against different cell treatments in correspondence to the applied DOX concentration (A = 100 µg and B = 30,0 µg), incubation periods (A = 4 hours and B = one hour) and Duty Cycle. A: dark grey = 25,0% DC and light grey = 20,0% DC. B: dark grey = 20,0% DC and light grey = 10,0% DC.
### Table 4.2: Statistical analysis of the different test samples executed with a Student’s t-test.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>p = 0.0751</td>
<td>p = 0.000885</td>
</tr>
<tr>
<td>B</td>
<td>p = 0.0315</td>
<td>p = 1.36 * 10^{-10}</td>
</tr>
<tr>
<td>C</td>
<td>p = 0.000137</td>
<td>p = 1.14 * 10^{-11}</td>
</tr>
</tbody>
</table>

Besides these four tests, a 12-well plate experiment was set up as cited in section 3.6.4 to test the inherent toxicity of the liposomal formulations on the cells, so no microbubbles were used in this test. The data are shown in figure 4-6 and p-values are listed in table 4.3.

The first test was executed with a concentration of 10.0 µg DOX per well. No significant difference is seen for the Doxil treatment between the 45 minutes incubation period (73.1% cell viability) and the four hours incubation (71.1% cell viability), whereas the Thermodox treatment indeed shows a significant difference between the two incubation-time settings (55.3% against 46.2% cell viability). One can see a significant difference as well between the treatments with 45 minutes incubation time and those with four hours incubation time, which are more toxic.

The second 12-well plate test was executed with a concentration of only 5.00 µg of DOX per well. Here, the Doxil treatments at both incubation periods show to be significant (93.3% cell viability against 77.6%). Again, Thermodox is more toxic for the cells and both treatments differ significantly (78.9% against 59.6 % cell viability). One can see a difference in cell viability percentage in relation to the concentration: the higher the DOX concentration, the less cell viability is seen. Besides this, Thermodox shows to be more toxic in-se in both experiments than when the Doxil liposomes were used.
Figure 4-6: The percentages cell viability plotted against the different treatments in a 12-well plate. The dark grey color represents 10,0 µg DOX per well, the light grey bars represent 5,00 µg DOX per well.

Table 4.3: Statistical analysis of the different tests on the 12-well plate executed with a Student’s t-test.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DOX concentration per well (µg)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxil 45’↔Doxil 4h incubation</td>
<td>10</td>
<td>0,596</td>
</tr>
<tr>
<td>Thermodox 45’↔Thermodox 4h incubation</td>
<td>10</td>
<td>0,000262</td>
</tr>
<tr>
<td>Doxil 45’↔Thermodox 45’ incubation</td>
<td>10</td>
<td>0,000190</td>
</tr>
<tr>
<td>Doxil 4h↔Thermodox 4h incubation</td>
<td>10</td>
<td>1,81*10⁻⁷</td>
</tr>
<tr>
<td>Doxil 45’↔Doxil 4h incubation</td>
<td>5</td>
<td>2,60*10⁻⁵</td>
</tr>
<tr>
<td>Thermodox 45’↔Thermodox 4h incubation</td>
<td>5</td>
<td>4,18*10⁻⁷</td>
</tr>
<tr>
<td>Doxil 45’↔Thermodox 45’ incubation</td>
<td>5</td>
<td>0,000138</td>
</tr>
<tr>
<td>Doxil 4h↔Thermodox 4h incubation</td>
<td>5</td>
<td>6,20*10⁻⁷</td>
</tr>
</tbody>
</table>
4.2 NANOEMULSIONS

As noted before, in order to evaluate the nanoemulsion phase-shift, the nanoemulsion particles were appraised pre- and post heating.

To start, the DLS evaluation was executed before heating as described in section 3.7.3.1. To create the curve, the number of particles was plotted against the size diameter and the results are shown in figure 4-7. The first measurement (in green) shows an average size diameter of more than 500 nm for most of the particles. The second measurement (in red) shows already an average diameter around one micron.

![Size Distribution by Number](image)

**Figure 4-7:** The DLS curve in which the number of particles (%) is plotted against size diameter (nm).

The particles were analyzed before heating by SPT as well. The frequency was plotted against the diameter of the particles and these results are shown in figure 4-8. An average diameter of approximately one micron is seen here as well.

![Frequency vs Diameter](image)

**Figure 4-8:** The SPT analysis curve, in which frequency (%) is plotted against diameter (nm).
A third test was a pre- and post-heating experiment executed with the Electrical Sensing Zone method. The curves were created by plotting the volume percent of the particles against the particle diameter and the results are shown in figure 4-9. When one studies the pre-heating curve, only one noticeable maximum peak for the particle size is seen, which is situated around 10,0 micron. The post-heating curve shows two peaks, one around 8,00 micron and one at 25,0 micron. Besides these results, the first peak of the post-heating experiment is slightly lower compared to the maximum peak which is seen before heating.

Figure 4-9: The Electrical Sensing Zone curve, in which volume (%) is plotted against particle diameter (µm). Pre-heating results are shown in black and post-heating results are shown in red.
5. DISCUSSION

5.1 LIPOSOMES AND MICROBUBBLES

The results of this thesis clearly show that thermosensitive liposomes loaded on microbubbles allow a significant increase in drug delivery efficiency, as shown by cytotoxicity experiments on BLM melanoma cells. This increased cytotoxicity is mainly seen when ultrasound is applied and can be declared by two factors: an increased DOX release out of the thermosensitive liposomes and more inherent toxic properties of the Thermodox formulation.

First, the increased DOX release out of the thermosensitive liposomes can be linked to the less stable nature of the TSL-microbubble drug delivery systems. Thus, if they are subjected to ultrasound exposure, the cavitation process of the microbubbles takes place, causing sonoporation and finally results in a more efficient DOX release. Moreover, focused ultrasound induces an increase in temperature as well, which affects the phase-transition of the thermosensitive liposomes’ bilayer membrane and hence leads to permeabilization of the membrane, which enhances DOX release as well.

Secondly, the thermosensitive liposomes own more inherent toxic properties than their Doxil counterparts, which was proved in the 12-well plate experiment. The increased inherent toxicity can be clarified by the incorporation of lysolipids in the thermosensitive liposomes’ bilayer. These lysolipids themselves are toxic because of their detergent properties. For this purpose we reduced the contact time between the cells and the liposomes to one hour, to really observe ultrasound related effects.

These two effects together prove that thermosensitive liposomes loaded on microbubbles allow a significant increase of drug release which is translated into a significant increase in drug delivery efficiency. Besides this, we have to consider the inherent toxic properties of the lysolipids as well.
5.2 NANOEMULSIONS

The two pre-heating size measurements of the nanoemulsion particles all showed average diameters around one micron. This is not in correspondence with what we expected to see because the nanoemulsions were extruded through a filter with a size cut-off of 200 nm. Are these large diameters due to the use of impure or inappropriate materials during the preparation of the emulsions or are there other, unidentified processes involved?

Post-heating experiments showed us that indeed larger particles were detected after the heating process. Smaller particles disappeared, which are believed to coalesce in order to create the larger particles. But this size-measuring assay has its drawbacks as well. In our protocol, only particles with sizes starting from approximately one micron could be detected, however we dealt with nano-sized particles, which hence could not be detected properly. This leaves the question if other methods can be found to evaluate the size of post-heating particles.

In general, the drawbacks and questions that raised with all tests, force us to consider the optimization of the nanoemulsion preparation and evaluation.
6. CONCLUSIONS

In this work we showed that we were able to produce thermosensitive liposomes. After attaching these thermosensitive Doxorubicin-containing liposomes onto the surface of ultrasound responsive microbubbles, we subjected them to ultrasound exposure. The experiments resulted in a significant increase in DOX release out of the Thermodox liposomes compared with traditional Doxil liposomes.

Cytotoxicity experiments as well revealed a significant increase in cytotoxicity upon ultrasound application if these thermosensitive liposomes were loaded on the surface of bubbles compared with Doxil liposomes.

We measured pre- and post-heating sizes of the nanoemulsion particles as well. Pre-heating, we could not detect nanoparticles. Post-heating experiments detected the expected large micron-sized particles. Nevertheless, the preparation procedures have to be optimized and we need to consider other methods for size-evaluation of the nanoemulsions.

In general we can conclude that thermosensitive liposomes linked to microbubbles indeed show significantly more cytotoxicity and are able to release their content more efficiently. The nanoemulsion evaluation still struggles with some problems and has to be optimized in further experiments.
7. LITERATURE AND REFERENCE LIST


(34) Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. Oncology 2005;69 Suppl 3:4-10.


Websites:

http://members.chello.nl/rwielens/ (12-03-2012)

http://www.echopraktijkzuid.nl/images/termijnecho3.jpg (26-03-2012)

http://prostate.tju.edu (26-03-2012)

http://stemcelllab.tripod.com/labEquip.htm (10-04-2012)
Internationalization at home: evening lectures

1. Improving adherence: from research to policy to practice by Nick Barber

During the Victorian age, pharmacists had to solve people’s problems, because their knowledge of science made pharmacists wise and trustable persons. The past decades, pharmacists were more seen as glorified salesmen, who paid less attention to side-effects of medicines. The biggest error which is made when medicines do not work, is non-adherence. Adherance is the most important subject to reduce harm from medicines. It is proven that when there was a talk in the pharmacy between patient and pharmacist about how the medicines work etc, the patients beliefs were significant shifted. This talk, before and after the treatment, creates a stronger band between the patient and the pharmacist. Hence, the patient will be more willing to improve his adherence. It is recommended to every pharmacist to have this talk with his patients.

We already discussed this subject in the course “Pharmacotherapy”, and it is indeed a very “hot topic”. I will absolutely try to talk to the people during my internship in the pharmacy, because I strongly believe in these improvement–rules.

2. Access to quality medicines in resources limited setting by Rafaella Ravinetto and Benedetta Schiavetti

Low income countries struggle with financial support for providing their citizens with approved medicines. The major problem here is money. First, only the government and the UN helped with financial assistance, nowadays NGO’s are taking a large part for their account. Other problems can be found in the lack of research and development for neglected diseases, who are most found in these regions, patents and monopolies. The distribution of medicines in the third world also causes problems. Here, solutions can be found in stock management and improved distribution flow. The quality of medicines have to be assured as well. The WHO has developed a pre-qualification program for these countries. The WHO visit manufacturing sites and list the medicines which have been approved. Therefore, the WHO does the work where the registration unit of the government cannot come to. But besides these efforts, still an enormous gap has to be filled between the North and the South when it comes to quality medicines access.
It was a good choice to focus our attention on this topic, because this is often forgotten in our education. It showed me yet another field of Pharmacy which I wasn’t really aware of and which found my full attention.

3. Pfizer forensic laboratory – EMEA region by Wendy Greenall

Counterfeit medicines are widely distributed in this current society via illegal internet sites. These illegal products barely consist of active compounds. They are made in unsanitary conditions and in massive production capacity. But why are counterfeit medicines produced? First of all, there is a low risk involved in manufacturing the “medicines”. Secondly, they are easy distributed and the producers can profit of a lack of patient awareness. Lastly, massive amounts of money can be made in this illegal business. Pfizer has its own counterfeit medicines laboratories to investigate false products. They make use of different forensic instrumental techniques to find where the problem is situated. But, no technique will identify all counterfeits. To go against these falsifications, Pfizer develops every two years new security features for their official products.

This lecture had potential to be interesting, if it would presented in a better way. Due to the insufficient communication of Mrs Greenall, it was hard to keep attention.

4. Computational chemistry in drug discovery: Can we improve productivity and reduce attrition? by Alexander Alex

To develop a drug from the first right molecule until the end of the production process, costs already one billion dollars. This shows that drug discovery is a very complex, expensive and time-consuming process. An explanation for this complexity can be found in the interaction of a drug with a protein for example, where the drug should be binding to. Drug research needs to become more productive, and here computational design can assist by shorting and reducing cycle time in discovery. The drug discovery process could be fastened if computer programs could be developed that could forecast if molecules would interact with a certain receptor. There are already “virtual screening” methods developed that enhance hit finding in drug discovery. Computational methods can assist in highlighting potential attrition risks. Computational design in the pharmaceutical industry will become even more important in the future.
This last lecture was a good alternation with the previous ones, which were found more in the “Pharmaceutical Care” direction. Most of the topics were already discussed in courses over the years, but it was a good to “refresh” this knowledge and handle it more deeply.