Preparation of light-sensitive ICG-Doxorubicin liposomes and developing of Quasi-Vivo® -based two-cell model for drug efficacy and toxicity testing.

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Tiivistelmä/Referat – Abstract

Traditional 2D cell cultivating vessels and experimental models cannot often simulate natural chemical and physical environment of different cell types. For example, availability of oxygen, chemical gradients, messaging molecules, fluid pressure, flow and surface topography are factors that may affect significantly in cell differentiation, growth, cellular structure, and metabolism. Modular bioreactors like Quasi-Vivo® -system can be used to simulate these factors.

Liposomes are particles of phospholipid bilayer with aqueous space enclosed within. They can be modified in numerous ways, like loading them with hydrophobic and hydrophilic molecules, changing their transition temperature or coating them according to different needs. Doxorubicin is effective and widely used cytostatic agent, but when administered as a free drug it has often severe side-effects, like cardiotoxicity.

Goal of this thesis is to determine appropriate manufacturing parameters and verify adequate shelf-life of ICG-Doxorubicin liposomes, that they are applicable for future *in vitro* experiments. Then survival of HepG2 cell line under flow in Quasi-Vivo®-equipment is determined, after which A549 and HepG2 will be then combined into one two-cell model. Finally, a simple illumination experiment in this cell model with previously made liposomes is conducted, and the effect in whole system is examined.

Using protocol presented in this thesis it is possible to produce successfully and repeatedly liposomes with both ICG and doxorubicin encapsulation over 70%. Their shelf-life was at least 14 days when stored in 4°C protected from light. This was determined to be sufficient for *in vitro* testing.

Cultivating A549 and HepG2 cell lines combined in the same system with shared media and fluid flow conditions was successful. Neither of the cell lines show significant difference in viability when compared to static control. When light-activating liposomes are administered to the system and then illuminated, from preliminary results we can see significant difference in drug effect. Both illuminated chambers and off-target chambers connected via Quasi-Vivo® show increased suppression, which shows promise that this *in vitro* model would be useful for future experiments.

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bioreactor, multi-cellular model, flow, liposome, doxorubicin, light-activated

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Valoaktivoituvien ICG-Doksorubisiini-liposomien valmistaminen ja Quasi-Vivo® -pohjaisen kaksisolumallin kehittäminen lääkkeiden teho- ja toksisuuskokeisiin

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Tiivistelmä/Referat – Abstract

Perinteiset 2D-solunkasvatusmenetelmät ja kokeelliset alustat eivät usein pysty simuloimaan eri solutyyppien luonnollista kemiallista ja fysiologista ympäristöä. Tekijöitä, jotka voivat vaikuttaa merkittävästi solujen erilaistumiseen, kasvuun, solunsisäisiin rakenteisiin tai metaboliseen aktiivisuuteen ovat esimerkiksi hapen saatavuus, viestiaineet, kemialliset gradientit, paine, nesteen virtaus ja alustojen topografia. Modulaarisia bioreaktoreita, kuten Quasi-Vivo®-järjestelmää, voidaan käyttää simuloimaan näitä tekijöitä.

Liposomit ovat fosfolipidikaksoiskerroksesta muodostuvia partikkeleja, joiden sisällä on vesitilavuus. Niitä voidaan muokata monin eri tavoin, lataamalla niitä kuljettamaan vesi- tai rasvaliukoisia molekyylejä, muokkaamalla niiden transitiolämpötilaa, tai päällystämällä niitä eri tarpeiden mukaan. Doksorubisiini on tehokas ja yhä laajassa käytössä oleva sytostaatti, jolla kuitenkin vapaana lääkeaineena annosteltuna on vakavia haittoja, erityisesti sydäntoksisuus.

Tässä työssä tavoitteena on selvittää sopivat valmistusparametrit ja todeta riittävä säilyvyys valoaktivoituville ICG-Doksorubisiini-liposomeille, jotta niitä voidaan käyttää tulevissa *in vitro* kokeissa. Tämän lisäksi selvitetään HepG2 solulinjan selviäminen virtauksen alla Quasi-Vivo® -laitteistossa ja yhdistetään HepG2 ja A549 solulinjat yhdeksi kaksisolumalliksi. Lopuksi suoritetaan yksinkertainen valotuskoe aiemmin valmistetuilla liposomeilla tässä solumallissa, ja tarkastellaan, miten vaikutus näkyy koko systeemissä.

Liposomien, joiden ICG- ja doksorubisiini-enkapsulaatio on yli 70%, valmistaminen onnistuu esitetyllä protokollalla luotettavasti ja toistettavasti, ja nämä liposomit säilyvät käyttökelpoisina ainakin 14 vuorokautta säilytettynä pimeässä, 4°C lämpotilassa.

A549 ja HepG2 solulinjojen kasvattaminen ja yhdistäminen samaan laitteistoon yhteiseen kasvatusliuokseen virtauksen alle onnistuu, eikä kummankaan solulinjan kasvussa huomata eroa viljelyyn staattisissa olosuhteissa. Kun valotetaan laitteistoon annosteltuja liposomeja, huomataan alustavien tulosten perusteella merkittävää tehon lisäystä valotetussa järjestelmässä pimeään verrattuna, sekä valotetuissa kammiossa että niissä, jotka siihen on Quasi-Vivo® -putkiston kautta yhdistetty.

Avainsanat – Nyckelord – Keywords
bioreaktori, monisolumalli, virtaus, liposomi, doksorubisiini, valoaktivoituva

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1. Introduction

Developing new pharmaceutical products has been growing more costly and time consuming for a long time. Affordable *in vitro* studies can help to screen pharmacological effects of new molecules in early stages of development, but it does not yet describe *in vivo* efficacy. Results drawn from animal studies also often do not translate into human, because of natural differences in metabolism and kinetics between species. This leads to promising molecules and formulations to be withdrawn from further development due to adverse effects or lack of efficacy in clinical studies. In addition, animal studies often cause some extent of harm to subjects even when we comply to all of the codes of conduct created to ensure wellbeing of the animals. This has been viewed all the time more prevalently as a moral cost of drug development.

Traditional 2D cell models can be used to replace some basic animal models, but they have several downfalls that can have large impact when trying to apply the results into whole organism. Recreating of the real-life physiological environment in which the cells are present in the body is often difficult. Cell-to-cell interactions, cell-to-extracellular matrix interactions and physiological cues, for example, are often crucial for normal cell functionality. These are usually not present in traditional well-plate based *in vitro* settings which can lead to significant changes in cell phenotype and functions (Hamilton et al., 2001; Lodish et al., 2000).

When developing better *in vitro* models, a lot of focus has been directed in developing so called organ-on-a-chip (OOC) or multi-organ-on-a-chip (MOC) microfluidistic models (Y. Zhao et al., 2019). These models often consist two or more different cell lines, which are interconnected with shared medium flow. Different designs can be found as many as there are studies conducted, and number of organs simulated with single system can

vary from one to five and more. Organ models used in MOC systems can also be almost anything from traditional cell monolayers to biopsies from human subjects and small 3D organoids. Differences and similarities between separate designs are described more in detail later in this review, but the effect of shear stress and intercellular communication is often simulated with either passive or active movement of shared medium between cell chambers. These models allow us to understand better how cells respond to specific stimuli, which is not easy to monitor *in vivo*. In this review I describe what are the factors regarding physiochemical conditions to be taken into consideration when designing cell culture systems, and how the experimental system we are going to use in later toxicity and efficacy testing will respond to these needs.

2. Impact of physiological and chemical environment in cells

In multicellular organisms, cells have evolved to work with each other to ensure the survival of the organism. They are specialized to produce cell-type specific molecules, conduct specific tasks and support, but on the other hand limit, the growth of other cells. Long history of this evolution means that intercellular communication is crucial to cell survival and without it cells cannot usually grow and operate normally. In addition to direct cell-to-cell contact, information is also transferred via endocrine messaging, soluble messaging molecules like cytokines and hormones, and lately progress has been made to understand cell communication with exosomes and other vesicles (Gerdes & Pepperkok, 2013). In addition to internal signals, organisms always operate in real world conditions where physical interactions with their environment are never insignificant. Organisms and cells within must observe and react physical changes around them to be able to survive, and this has straight impact on cell biology, which then must be taken into consideration when culturing cells. Further I will discuss how shared media and natural-like fluid movement can provide significant benefits in cell culturing.

2.1. Chemical environment

To make cell cultivation in laboratory possible, cultures are often supplemented with serums like fetal bovine serum (FBS), usually with 10% (v/v). It contains necessary growth factors and hormones to keep many frequently used cell lines alive and proliferating. Still it does not fully resemble real communication with other cell types, and since it is harvested from bovine fetuses there is always an ethical concern related (Jochems et al., 2002). Although serum free medium is being developed and often encouraged, there is still benefits in using FBS especially when creating shared medium for several cell types. Using serums or other supplements might keep cells alive, but when intercellular signalling is made possible, additional benefits might be seen.

Great importance of these signals relates to for example cell differentiation from muscle progenitors to skeletal myoblasts or pericytes, depending on the signals received (Cappellari et al., 2013). Furthermore, cells like hepatocytes change their CYP-enzyme activity and expression depending on the cell-to-cell interactions (Hamilton et al., 2001). Changes in hepatocyte enzymatic activity can have a massive impact when screening drug toxicity or metabolism. When comparing viability of cardiomyocytes, cardio endothelial cells, and smooth muscle cells alone and in co-culture, a significant increase in cell viability has been observed when cells are cultivated together (Alias et al., 2018). This gives cell culture increased robustness for example against drug toxicity. Considering these factors, it seems often beneficiary to culture cells in more natural contact with other cells.

Availability of oxygen is often limiting factor considering cell viability and proliferation in static culture. When cultured in incubators with similar oxygen concentration as in atmosphere, fresh medium with no cells contains around 0,2 mM oxygen (Sbrana & Ahluwalia, 2012), but when oxygen consuming cells are added, oxygen level falls rapidly relative to depth of culture medium. In hypoxic conditions viability of different cells, for

example hepatocytes, falls radically within 24-48 hours (Smith & Mooney, 2007). Flow conditions will provide great benefits and reduce the possibility of oxygen deficit in cell culture, when gas transport is aided with circulating medium (Mattei et al., 2014, Figure 1). Hypoxic cell cultures can be used to investigate different pathologies like solid tumours but cultivating mammal cell lines still needs more often sufficient gas exchange. Availability of oxygen, chemical gradients, and possibility to communicate can give benefits to cell culture viability, their development and activity which could often be beneficial in drug research. When combining these with relevant physiological factors, it is possible to take steps towards life-like cell models.

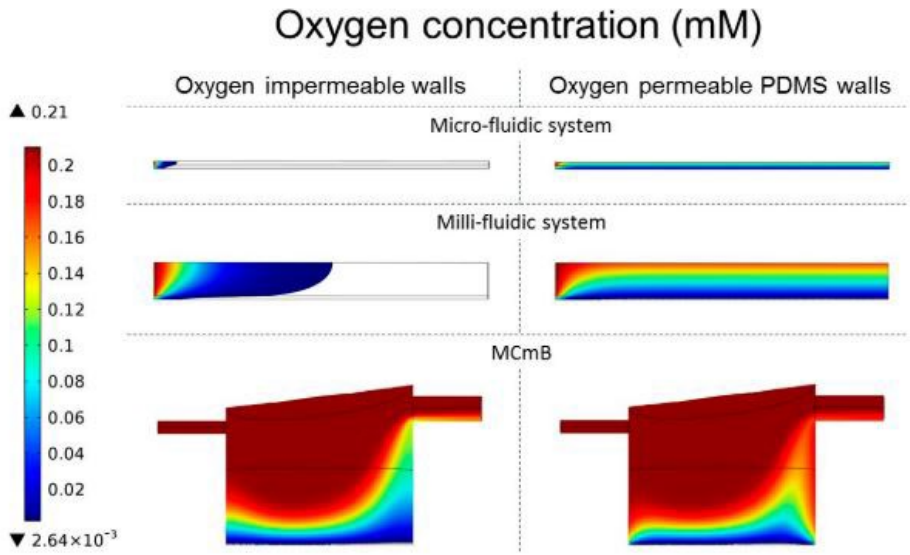


Figure 1: Significance of geometry, fluid dynamics and material permeability for oxygen supply. As demonstrated, in this Multi-Compartmental Modular Bioreactor (MCMB) cell culture has significantly improved oxygen supply. With permission from Mattei et al. (2014).

2.2. Physiological conditions

Living cells are naturally exposed on different pressure, stretch and shear stress conditions, depending on the area where they are located in body. Due to this, different

cell types require different physical conditions to be able to grow and perform as intended. As it seems logical, especially endothelial cells are *in vivo* under constantly changing fluid pressure and stress, and it has been established that endothelial cells thrive and their phenotype resembles natural state best, when exposed to changing physical conditions (S. Zhao et al., 1995). For example, they are organized more regularly, cytoskeleton is further developed and metabolism changes to support efforts to maintain cell layer integrity (Davies & Tripathi, 1993). Effect of shear stress has been under interest especially when investigating vascular endothelium, and how it relates to for example atherosclerotic pathogenesis (Cunningham & Gotlieb, 2005). In hepatocytes, it has been demonstrated that prolonged exposure to shear stress *in vitro* increases their ability to metabolize ammonium and synthetize for example urea, albumin, and alanine aminotransferase (Park et al., 2008; Tilles et al., 2001). When culturing osteoblasts under shear stress, it does seem to be promoting actin and integrin formation and construction of organised structures, which might be important factor in new bone tissue formation (Pavalko et al., 1998).

What could be even more relevant to pharmaceutical studies, is the fact that shear stress also can change pharmacologically important enzymatic activity on cells. Increased shear stress is shown to reduce angiotensin converting enzyme activity in pulmonary arterial endothelial cells, depending on the strength and duration of the exposure (Rieder et al., 1997). Appropriate medium flow when cultivating hepatocytes, can lead to increased expression of metabolic enzymes, like CYP3A4, CYP2B6 and UGT2B7, sometimes up to 30-50-fold, compared to the reduced appearance in static conditions (Shvartsman et al., 2009; Vinci et al., 2011).

Mechanical cues, like shear stress, growth surface topography and other physical interactions cause cell stress and can also have their effect on the cell differentiation. In flat surfaces shear stress can induce endothelial differentiation, especially via cytoskeletal tension and cell contractions (Sonam et al., 2016). But when pluripotent

stem cells are cultured in a stirred bioreactor shear stress could help to maintain pluripotency genes (Gareau et al., 2014), which shows that cell differentiation is complicated process and though shear stress has its significance, it is only one environmental factor among many. Examples above show how important physical conditions are for cell development and survival. Both the chemical and physiological conditions are possible to be simulated with novel cell cultivation systems described in next chapters.

3. Organ models and fluidic control in milli- or microliter scale

To be able to combine the benefits of intercellular communication and dynamic fluid movement, new methods have to be developed to integrate separate cell cultures and organoids within same system. This is often not as fast forward as some might first think, since to be able to both make models to resemble real life interactions in scale, while simultaneously keeping the system easy to use and cost-effective, researchers must overcome some significant biological and physical challenges.

3.1. Model scaling

When building a multi-organ model, often must be considered how the model would be most relevant to current research. Some could consider that perfect miniature of human organ systems in relative scale would be the best option, but it would have some significant design challenges. In fact, this would be practically impossible as described in paper by Wikswo et al. (2013), because of allometric scaling regarding to not only relative organ size, but the blood volume it would need to support its oxygen needs. Table 1 presents how using established allometric scaling laws dimensions of organs (M_o) and their portion of blood supply (% M_b) changes, which leads to up to 10-fold

relative differences of metabolic needs depending on the organ (1000 mHu/Hu). It shows how different regarding to energy and oxygen supply human-on-a-chip would need to be, not yet even considering how this would be achieved in laboratory conditions. In addition, sizes of single cells are not scalable, which means that for example endothelial monolayers would quickly be unproportionally thick.

Table 1: Different organ weights (M_0) and proportion of blood supply (% M_b) scaled allometrically. Scaling is done from human (Hu) to milliHuman (mHu) using established primate scaling laws, and what would relative organ size need to be in milli scale compared to real human body (1000 mHu/Hu). (Wikswo et al. 2013)

	Hu M_b = 60kg		**mHu M_b = 60g**		**1000 mHu/Hu**
Organ	**M_0, g**	**% M_b**	**M_0, g**	**% M_b**	
Liver	1500	2.5%	2.4	4.0%	1.6
Brain	1300	2.1%	13	22%	10
Lung	4600	0.8%	0.39	1.2%	1.5
Heart	280	0.46%	0.34	0.57%	1.2
Kidneys	2200	0.37%	0.54	0.91%	2.4
Pancreas	83	0.14%	0.15	0.26%	1.9
Spleen	49	0.081%	0.14	0.23%	2.8
Thyroid	15	0.025%	0.01	0.01%	0.44
Adrenals	9.3	0.016%	0.07	0.12%	7.9
Pituitary	0.49	0.001%	<0.01	0.01%	9.1

Although allometric scaling has been used even with surprising success for example estimating human first dose (Boxenbaum & DiLea, 1995), in milli- or microscale modelling it is not especially useful. Instead, when building small organ models, more important is to consider what are the important physiological functions to be examined and add on top of those adequate computational models and chemical analysis.

3.2. Fluid control

Fluid volume in system poses further challenges, since too large volumes of blood surrogate will dilute paracrine and endocrine signalling between cells, which would then reduce benefits from multi-cellular systems (Faley et al., 2008), and when investigating effect of metabolites large fluid volume would dilute them non-effective (Oleaga et al., 2018). But then on the other hand small, microlitre-scale volumes would cause great challenges for pumps integrated in these systems, since most commercially available pumps have dead space orders of magnitude larger than the volume of entire cultivation system would be (Wikswo et al., 2013).

Micro-scale fluidics also have other problems, since when diameters in cultivation chambers shrink, it leads to high surface-area to volume ratio and increased shear stress towards cells which evidently results in decreased viability (Tilles et al., 2001). When trying to avoid this problem by limiting flow rate, it cuts down oxygen and nutrition supply to cells; very significant with for example hepatocytes or cardiomyocytes and their large energy consumption (Mattei et al., 2014). Small fluid volumes can also lead into common formation of air bubbles, when surface forces are more significant compared to volume. This shows again that balance in fluid dynamics must be found within experimental priorities, and correct system design depends on the parameters measured.

Microfluidic systems can be most useful when we want to investigate effects of well-defined single factors in cell behaviour (Figure 2). For example, some systems have been developed that are able to create really finely tuned chemical gradients within a space of a fraction of a microlitre, and thus allowed scientists to study for example IL-8 gradient on neutrophil chemotaxis, growth factors on neural stem cell differentiation and cell reaction to viral infections (Geun Chung et al., 2005; Jeon et al., 2002; Walker et al., 2004). Small scale systems also allow better control in forces applied to single

cells, for example with precisely controlled laminar flow (Hudson et al., 2004) or magnetic systems allowing us to physically interact with the cells from outside the system (Sniadecki et al., 2007).

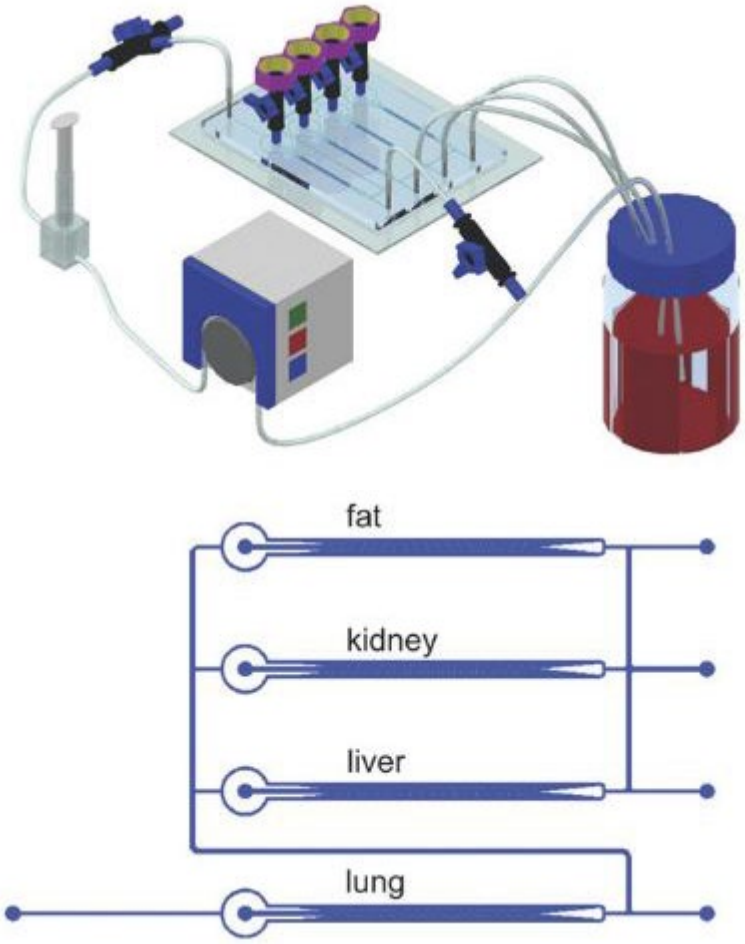


Figure 2: An example of an MOC model in microscale. Four organ models are incorporated within one chip and perfused with circulating media. Modified from Zhang et al. (2009)

3.3. Multi-Compartmental modular Bioreactors

Smaller the scale of the system, more significant are the problems related to small volumes. High surface-area to volume ratio will affect the cells as described, and since cultivation systems are nowadays often moulded using polydimethylsiloxane (PDMS) or

other elastomers, their tendency to attract small hydrophobic molecules becomes more significant (Mazzei et al., 2010). To eliminate these issues, so called Multi-Compartmental Modular Bioreactors (MCMBs) have been developed. These reactors are more versatile than often single-purpose microfluid systems, and modifiable for the needs of different experimental settings, thus allowing to optimise parameters for longer and more complex studies (Orr & Burg, 2008; Schuerlein et al., 2017). Millilitre-scale systems are also significantly easier to handle, and even to be build in-house as Schuerlein et al. (2017) demonstrated. MCMBs have been used to investigate intercellular crosstalk via shared media (Guzzardi et al., 2011), model basic glucose and lipid metabolism interplay between hepatocytes, endothelia and adipocytes (Vinci et al., 2012) and demonstrating how co-culturing hepatocytes and umbilical cord endothelial cells enhances both survival and function of these cells (Vozzi et al., 2008), for example.

3.4. Quasi-Vivo® as a platform.

Commercially available MCMB system that is going to be used in this thesis' experimental part is Quasi-Vivo® (further QV) from Kirkstall Ltd (York, North Yorkshire, UK, Figure 3). It consists of separate cell culture chambers, which are linked with tubing simulating blood circulation, and with peristaltic pump to provide continuous fluid flow transporting nutrients, oxygen and waste products in steadily fluctuating movement (Sbrana & Ahluwalia, 2012). Pump velocity can be adjusted, so amount of shear stress cells are subjected to can be changed according to situation. During this thesis QV will make it possible to simulate target and off-target drug effect, while possibly providing cells with more natural growth environment and reliable results.

System supports simple monolayer cultivation, as well as more complex 3D-structured cultures when they are seeded for example polyvinyl alcohol or collagen based polymer scaffolds (Alias et al., 2018). Culture chambers are manufactured from PDMS which is biocompatible elastomer with high oxygen permeability. Material is chemically inert but

binds non-specifically into small hydrophobic molecules like drugs, which is something to consider especially when experimenting with potent drugs and small dosages (Mata et al., 2005). QV could be used to simulate human body functions with several cell models, where paracrine and endocrine communication are possible within a scale of a normal 24-well plate. Also, drug treatments introduced to the system or their metabolites travel from cell chamber to another, which gives possibility to do new kinds of efficacy and toxicity testing. Culture chambers are modular which makes it easy to move and transform experimental setting, and they can be connected in series or in parallel (Sbrana & Ahluwalia, 2012). System does not have integrated gas or heat control, so it must be maintained inside cell cultivation incubator. This might lead some handling challenges, but with proper training system and cell cultures can be operated without contaminations.

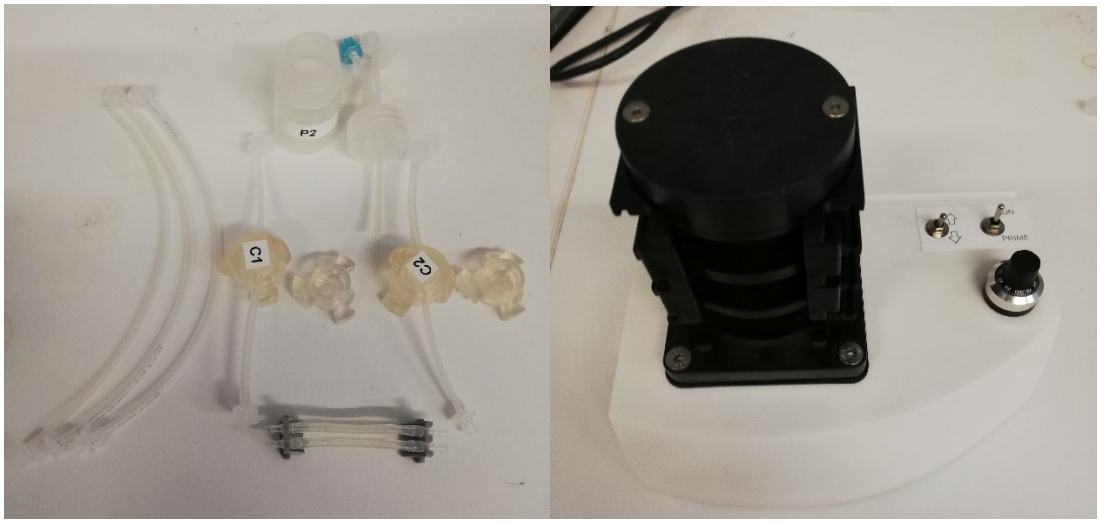


Figure 3: Parts of the QV system used in the experiments. On left presented PDMS tubing and culture chambers, and plastic-made media reservoir on the top. In this thesis, two chambers connected in series with reservoir were used. On the right, electric peristaltic pump which could supply in theory up to 12 chamber systems.

4. Creating a shared environment for two cell lines

In this thesis I will be working with a two-cell system cultivated in QV system presented above (Figure 3), which could be used to efficacy and toxicity testing for different drug molecules and formulations. Cell lines are chosen according to their known robustness and current availability in our laboratory. Cell lines from varying sources have different needs regarding to growth media composition, and unsuitable media could lead to decreased growth or viability. Too fast fluid movement, which would provide increased shear stress, can also decrease cell viability, which will be discussed further on this review. Growth media which will be used in the *in vitro* study will be chosen by suggestions from the literature, and suitable cultivation flow rate will be determined experimentally.

4.1. Cell lines used in future experiments

4.1.1. A549 cell line

A549 is human adenocarcinoma cell line, which forms confluent alveolar epithelial type II (ATII) like monolayers in cultivation (Figure 4). They are usually maintained either in simple medium like Dulbeccos's Modified Eagle's Medium (DMEM), or then more physiologically relevant medium like Ham's F12 (F12) which could lead to more ATII like differentiated phenotype (Cooper et al., 2016), supplemented with 10% (v/v) FBS and, when deemed necessary, 1% (v/v) penicillin-streptomycin (PenStrep) (Zuchowska et al., 2017). Cells need to be subcultured before reaching confluency, monolayer can be detached with Trypsin-EDTA solution. Cells are incubated with humid environment in 37°C and 5% CO_2.

Although A549 is an epithelial cell line, it has been noted that it could be somewhat sensitive to shear stress, and 20 µl/min flow did decrease the viability of A549 cells in spheroid culture (Zuchowska et al., 2017). Quite opposingly, other literature is suggesting, that even rather high flow rate 100 µl/min did not decrease viability in flat-surface culture (Mahto et al., 2014).

This might be due to difference in culture medium, since Zuchowska et al. (2017) used DMEM which should lead towards more mesenchymal-like differentiation, compared to DMEM/F12 used in Mahto et al. (2014) experiment, which has been demonstrated to cause cells to express more epithelial-type phenotype (Cooper et al., 2016; Selenius et al., 2019). Mesenchymal-type cells are more sensitive to shear stress, and it could cause arrest of cell cycle or even lead to cell death (Luo et al., 2011; Zuchowska et al., 2017).

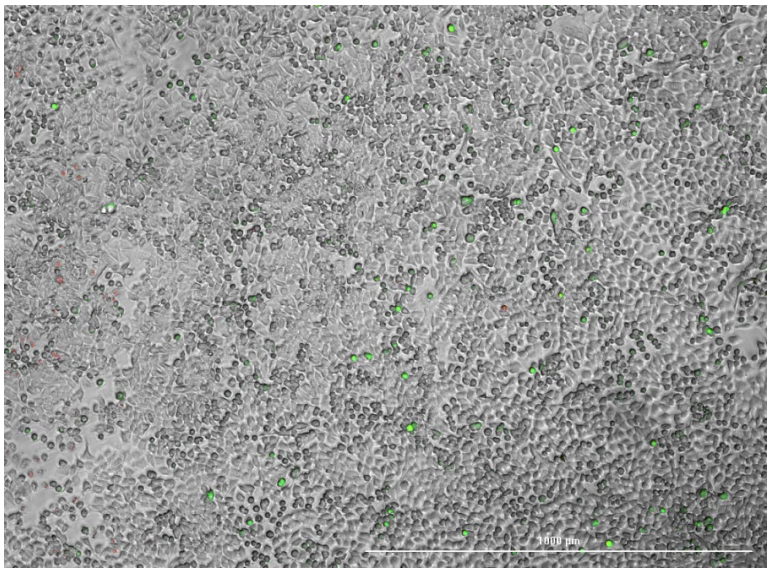


Figure 4: A549 cell culture. A549 forms dense endothelial monolayer when cultivated. Image captured after Live/Dead™ kit treatment with Cytation 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT, USA).

4.1.2. HEPG2 cell line

The human hepatoma HEPG2 is an adherent cell line that grows as monolayers in small aggregates (Figure 5). They are highly differentiated cells and express many genotypic features similarly to normal liver cells (Sassa et al., 1987). Growth medium can be DMEM, or alternatively Eagle's Minimum Essential Medium (MEM), Roswell Park Memorial Institute medium (RPMI-1640) or F12, supplemented with 10% (v/v) FBS and PenStrep when necessary (Gerets et al., 2012; Selenius et al., 2019). Cell incubator should be humid 37°C with 5% CO_2.

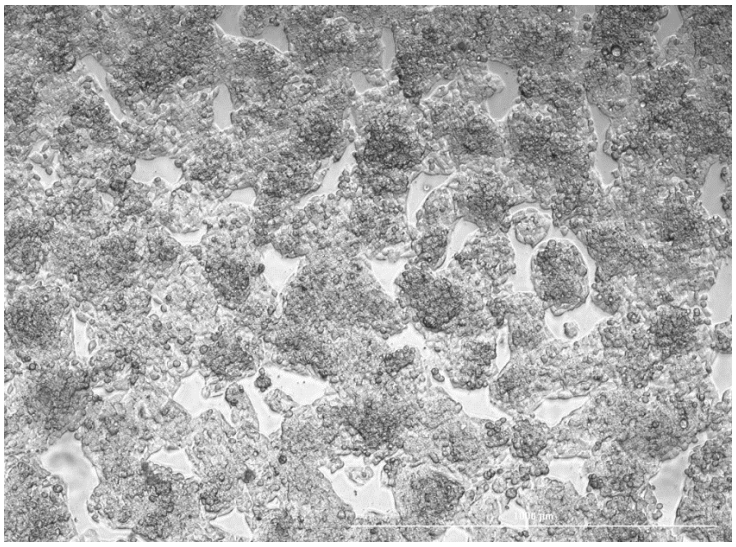


Figure 5: HepG2 cell culture. HepG2 tend to form small clusters and spheroids especially visible before reaching confluency. Picture is captured with similar system described in previous figure.

Although HEPG2 is extremely widely used cell line in drug toxicity testing, it must be noted that its sensitivity to detect toxicity in compounds that require metabolic activation is limited, due to its reduced expression of metabolic phase I enzymes (Xu et al., 2004). This is not a major issue when research is focused mainly liver toxicity, but in metabolite toxicity testing more preferable options would be primary hepatocytes or

HEPARG cell line which consists of both hepatocyte-like and biliary-like hepatic carcinoma cells (Gerets et al., 2012).

According to literature, HEPG2 cell line benefits significantly from dynamic culturing conditions, compared to static: their growth rate, metabolism, CYP1A enzymatic activity and viability was improved with fluid flow rates of 10 µl/min and 25 µl/min, when there was no significant difference between these flow rates (Baudoin et al., 2011; Ye et al., 2007). Of course, excessive shear stress starts to damage cells and reduce their activity, optimal flow rate has to be determined separately according to equipment used (Tilles et al., 2001).

4.2. Shared growth medium

Recommended medium used to culture A549 is according to suppliers DMEM or F12, and for HEPG2 it is MEM (*Recommended Media Types for Common Cells, Thermo Fisher Scientific*). From these media, DMEM contains up to four times more vitamins, amino acids, and glucose compared to MEM. F12 also contains double the amount of glucose compared to MEM, and it is supplemented with different set of vitamins, amino acids and metals, including for example vitamin B12 along with copper, iron and zinc sulfates (Arora, 2013; Selenius et al., 2019). It is also possible to buy on-shelf DMEM/F12 medium mixture, which is especially rich and complex medium that would support many different cell lines.

According to Selenius et al. (2019) when they compared different growth media for both cell lines, they noticed that with DMEM either cell lines had the fastest proliferation rate, and slightly larger ATP production (Figure 6), probably mainly due to higher glucose concentration and increased amounts of important amino acids like methionine and cysteine, these beneficial effects have been demonstrated before (Han et al., 2015;

Okuno et al., 2014). As discussed, A549 cells do change their phenotype according to medium used and with F12 they will express more epithelial-type genes, while with DMEM or MEM their phenotype is more mesenchymal-like. With HEPG2 similar changes are not observed.

Previous experiments with shared medium between A549 and HEPG2 in addition to other cell types have been conducted by combining each optimal media with each other, and then supplementing it with necessary components (Zhang et al., 2009). However, when we primarily cultivate only these two cell lines discussed, according to literature rather good results are to be expected from using DMEM supplemented with FBS.

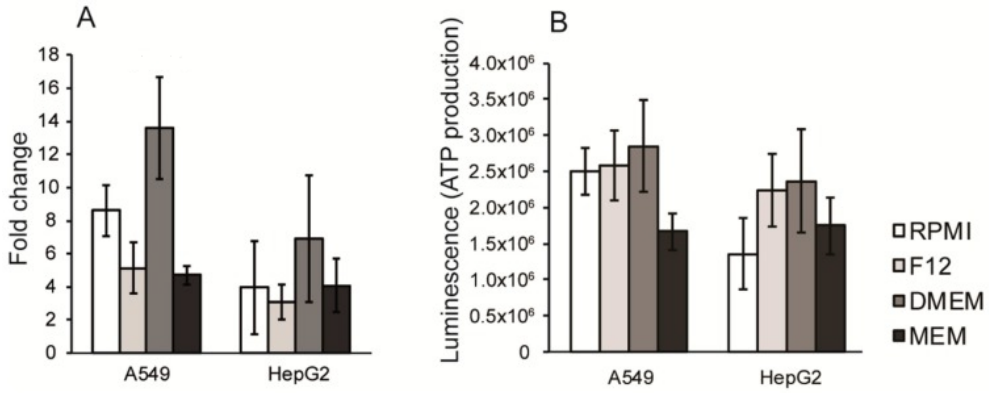


Figure 6: Growth of cell lines HepG2 and A549 in four different growth media. Results show that with both cell lines proliferation (A) and ATP production (B) is increased the most with DMEM, probably due to larger glucose and amino acid concentrations. Modified from Selenius et al. (2019).

5. Light-activating liposome vehicles with doxorubicin payload

In addition to investing viability and growth of these two cell lines in combined system, another goal of this study was to create a reliable protocol for manufacturing of light-reactive liposomal vehicles loaded with doxorubicin, after which combining these two for efficacy and off-target toxicity testing in new MCMB model. Further will be discussed

how combining liposomal formulations with mechanisms that would release the payload with external stimuli could be beneficial when treating serious diseases with therapeutics, that are efficient but highly toxic when administered systemically. MCMB model manufactured from PDMS used in our experiments would possibly provide suitable platform to test these formulations in pre-clinical phases.

5.1. Liposomal formulations

Liposomes are a nanotechnology drug delivery system, and one of the most well-known and investigated vehicle for improved and targeted drug delivery (Sercombe et al., 2015). By definition they are spontaneously assembling phospholipid vesicles consisting of one or more lipid bilayers that enclose aqueous spaces within (Ding et al., 2006). One of the unique properties of liposomes is that we can encapsulate both hydrophobic and hydrophilic molecules; hydrophobic molecules are trapped inside the lipid bilayer and hydrophilic molecules can locate in aqueous solution inside the liposome (Sercombe et al., 2015). For macromolecular delivery this structure is also beneficial, for example large protein and DNA payloads have been used successfully (P. Y. Liu et al., 2004; Monteiro, Martins, Reis, et al., 2014). In addition, release of small molecular drugs can be prolonged with implants incorporating liposomes, where drug is then released with help of external stimuli or just slowly during long periods of time (Monteiro, Martins, Pires, et al., 2014). In next pages several ways of modifying liposomes for different applications are described, which are also presented in figure 7.

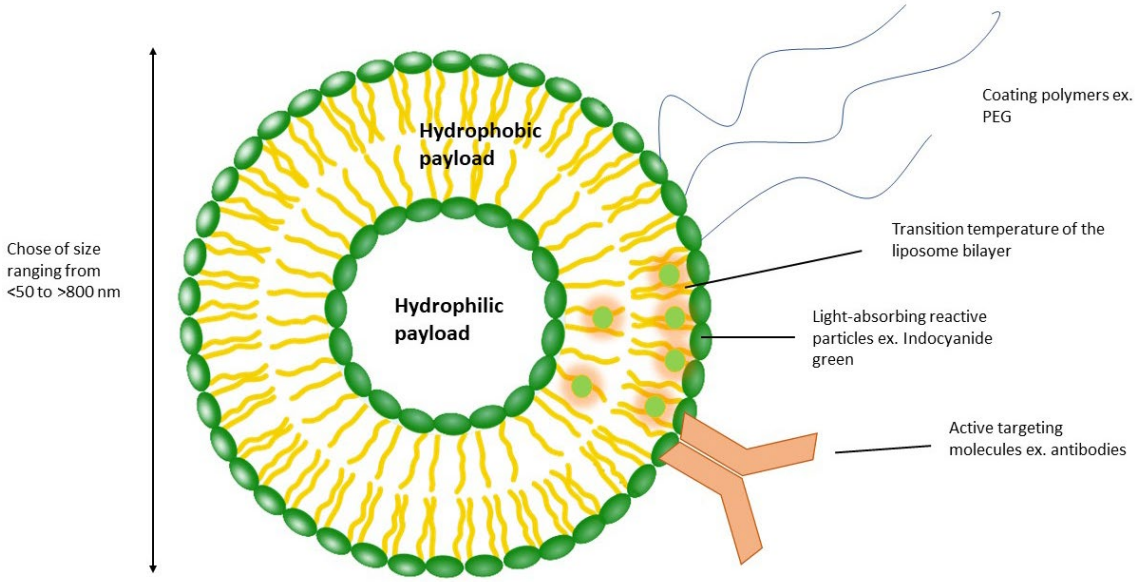


Figure 7: Graphic presentations of factors that liposomes can be modified by according to the needs of the application.

5.1.1. Liposome manufacturing

Great benefit of using liposomal carriers is that they are very modifiable. Preparation methods can vary greatly depending on the desired liposome size and payload. Most often process of making liposomes consists of following phases: drying out lipid film from organic solvents, rehydrating lipids with aqueous media, then possibly liposome size control phase by for example sonication or extrusion, purification with methods like dialysis or gel-permeation chromatography, and final product analysis (Akbarzadeh et al., 2013).

Liposome size can be controlled with relative ease and there are several techniques to ensure constant and predictable size distribution, which of most feasible in simple laboratory setting are sonication or extrusion (Berger et al., 2001; Dua et al., 2012). Often extrusion is more reliable way to control liposome size and avoid for example unwanted formation of multilamellar liposomes. With modern methods it is possible to

produce liposomes with great range of sizes, but often most suitable for pharmaceutical use are particles between sizes 50-400 nm (Etheridge et al., 2013).

5.1.2. Heat and light triggered drug release

Drug administering inside liposomes that undergo some changes when temperature is increased could be a great way for drug targeting. Liposomes could be made to release drug when body temperature rises, like during fever, or then by using external stimulation. Transition temperature (T_m) of lipid bilayer describes temperature below which lipid bilayer is closely packed and rather rigid gel of phospholipids, and transition through the bilayer is at least for larger molecules almost non-existent. When temperature approaches T_m, bilayer structure is starts to loosen up and payload starts to leak out (Chen et al., 2018), although sometimes it might be important to notice that permeability of liposomes might be already increased a little bit below T_m (Papahadjopoulos et al., 1973). By changing the phospholipid composition, T_m can be modified to be suitable for different implementations. Liposomes can be made to be for example stable in room temperatures, but to break down when administered into body (Sadeghi et al., 2019).

Heat-sensitive changes can also be triggered by external signals. Simplest ways would be using some method to warm up tissue itself where the effect of the drug is desired (Gasselhuber et al., 2012; Rossmann et al., 2017), but trying to avoid heat related tissue damage and make drug targeting more precise in relation to location and timing of the treatment, liposomes can be manufactured to react also other external stimuli, like light (Leung & Romanowski, 2012). For liposome to be light sensitive, some structural changes within have to be induced by light with correct energy (Lajunen, Nurmi, et al., 2016). Techniques have been developed that rely on photothermal conversion, where small light absorbing particles are included within liposome or incorporated in the bilayer (Leung & Romanowski, 2012). Produced heat after illumination then induces

disorder in liposome, and payload is released. Materials used to produce this effect can be molecular dyes like sulforhodamine or indocyanide green (Lajunen, Kontturi, et al., 2016), small metallic particles for example non-resonant gold nanoparticles (Paasonen et al., 2007) or plasmon resonance assisted release with larger metal particles or with full 'nanoshell' coating (Troutman et al., 2008). With these methods it is possible to reduce possible treatment phototoxicity, promote the use of more penetrating light wavelengths, and improve treatment accuracy while reducing the chance of free radical formation (Leung & Romanowski, 2012). Light could also trigger other changes in liposomes, wavelengths used and changes in the structure could be various. Conformational changes induced by light would cause bilayer to destabilise, photocleavage could cause amphiphilic molecules to become hydrophilic which would lead liposome disintegration, and light-induced polymerisation or depolymerisation will also lead to liposome destabilising (Lajunen, Nurmi, et al., 2016; Leung & Romanowski, 2012; Paasonen et al., 2007).

5.1.3. Protective coating

Liposomes are very modifiable as described, and their outer surface composition and coating can be changed according the needs of application. Reticuloendothelial systems are efficient at removing foreign particles from circulation, and in case of liposome formulations, clearance could be increased by using charged phospholipids (F. Liu & Liu, 1996) or reduced by using surface polymers like polyethylene glycol (PEG) (V. P. Torchilin & Trubetskoy, 1995). PEG coating gathers water on the surface of the liposomes, which prevents attachment of opsonins, and PEG also works as a steric hindrance preventing phagocytosis (Nunes et al., 2019; Sadzuka et al., 2002). Non-modified nanoparticles tend to cumulate in filtration organs, mostly in liver which can capture as much as 90% of systemic dose (Gustafson et al., 2015). Although PEG coating has been demonstrated to reduce for example doxorubicin cardiotoxicity (Rifkin et al., 2006), encapsulation might lead into other toxic effects. Doxorubicin liposomal formulation Doxil® can cause up to 50% of the patients palmar-plantar erythrodysesthesia (PPE), which is painful

dermatitis in limbs and peripheral skin areas (Lorusso et al., 2007). Prolonging liposomal half-life in circulation can aid significantly with drug targeting but can lead to unexpected adverse effects.

5.1.4. Passive and active targeting

Liposomal drug targeting can be more efficient than with free drug, especially in cancer treatment. Passive targeting of anti-cancer liposomes relies upon the fact, that tumor associated vasculature is not dense, but instead rather leaky, with significant gaps between endothelial cells (V. Torchilin, 2011). When systemic exposure is prolonged, liposomes pass larger number of times through cancer site and have larger chance to permeate this endothelial layer compared to healthy vasculature (V. P. Torchilin, 2007). Actively targeted liposomes on the other hand include some small molecular ligands, peptides or antibodies on the liposome surface, which should allow liposomes to better attach target cells and promote endosytosis (Egusquiaguirre et al., 2012; V. P. Torchilin, 2007). Experiments with some promising results have been conducted with for example ligands that bind into folate receptor (Low et al., 2007), transferrin receptor (Zhai et al., 2010) and Her2 receptor (Shmeeda et al., 2009) which all are found over-expressed in selected cancer types. Although active targeting might give some benefits related to cell recognition and attachment still several obstacles in drug delivery persists, like tumour heterogeneity, tight physiological barriers and enzymatic degradation, leading overall drug exposure in tumour area being often not as high as desired (Rosenblum et al., 2018). Here combining of passive targeting and controlled release come to play, since by increasing circulation time and simultaneously limiting drug exposure only on treatment site, it might be finally possible to significantly increase treatment efficacy and reduce side effects.

5.2. Drug-loaded liposomes for the experiments

5.2.1. Active pharmaceutical ingredient - Doxorubicin

Anthracycline drug doxorubicin (DOX) has been used as a chemotherapeutic agent since 1960, and it is used to treat several different solid tumours like sarcomas and breast- bladder- and thyroid cancer along with acute myeloblastic leukemia, lymphoblastic leukemia and small cell lung cancer (Johnson-Arbor & Dubey, 2007).

DOX mechanism of action is based on intercalating with DNA strands leading to inhibition of DNA and RNA synthesis, by preventing topoisomerase II -enzyme progression. Moreover, doxorubicin hydrochloride can cause oxidative stress, leading to further DNA and cell membrane damage (Thorn et al., 2011). DOX toxicity is also most often linked to its capability to generate free radicals, and toxicity has been demonstrated in several organ models, like hepatic, cardiac, and renal models (Oleaga et al., 2018; Shivakumar et al., 2012). Clinically most significant toxic effect is often considered to be cardiomyopathy, which seems to be mediated via upregulation of apoptotic receptors like TNFR1, Fas and DR4 (L. Zhao & Zhang, 2017). Multi-organ toxicity of DOX treatment can lead to lifelong impairment and increases risk for further health problems. It has been demonstrated in retrospective analysis, that incidence of congestive heart failure is up to 5 times higher with patients who have received treatment with >250 mg/m^2 DOX (Mulrooney et al., 2009). Moreover, DOX has been linked in several, sometimes fatal, hepatic complications, thus it is described to be likely clinically significant hepatotoxic agent (NIDDK, 2012).

Although some anthracyclines like epirubicin and idarubicin with reportedly reduced cardiotoxicity have been developed, DOX is still one of the most commonly used anticancer agents (Waterhouse et al., 2001). This has led in development of new formulations that could reduce adverse effects of treatment. Two DOX liposomal

formulations are already approved for use to affect the biodistribution of the molecule and decrease drug concentrations in the most vulnerable organs, especially in heart (Abraham et al., 2005). Liposomes have increased circulation time when coated with polyethylene glycol (PEG), which provides some passive targeting to solid tumours since particles have longer time to find their way through leaky vascular endothelium present in tumour areas. (Papahadjopoulos et al., 1991).

5.2.2. Liposome loading methods

Loading liposomes with payload molecules can be achieved passively or actively. Passive method often relies in thermodynamic distribution and results in low encapsulation efficiency (EE) unless the drug is lipophilic and attracted to stay in lipid bilayer (Sur et al., 2014) When using passive loading methods with hydrophilic molecules, best EEs possible to achieve are <50% and require methods like reverse-phase evaporation, which are not really feasible in commercial production (Szoka et al., 1978). Often more efficient methods are active loading procedures, meanly using pH gradients. For example, hydrating lipid film with acidic solution, like citrate buffer or ammonium sulfate, creates acidic environment inside liposomes, after which external phase can be changed to neutral, thus creating a pH gradient.

When manufacturing DOX liposomes, drug is unionized in neutral external solution, and when sulfate ions and protonated doxorubicin interact in internal acidic environment, they from bundles of insoluble fibres which then attracts more dissolved DOX inside liposome, leading at best >90% EE (Fritze et al., 2006; Gubernator, 2011; Li et al., 1998, Figure 8). Active loading techniques can be used with different drug molecules, when the properties of drug and used gradient are investigated and carefully matched.

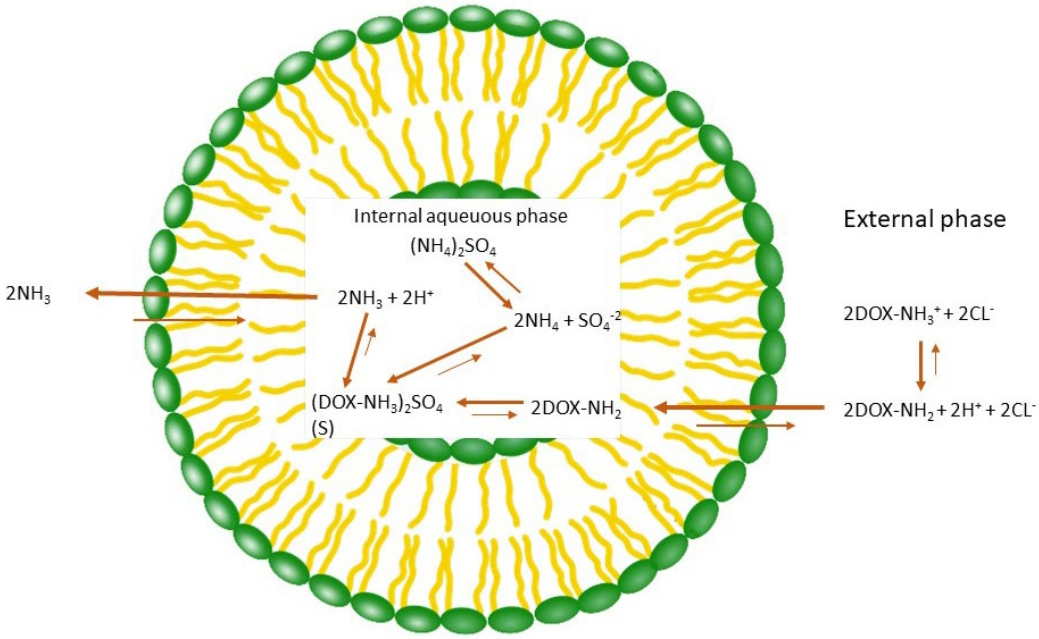


Figure 8: Graph of the process of actively loading doxorubicin inside liposomes using ammonium sulfate gradient.

6. Premise of the study

Pre-clinical pharmaceutical development relies on basically three main methods of research: in silico, *in vitro* and *in vivo*. Techniques in transforming results from simulations to *in vitro* models are quickly developing further, but when it comes to replicating results of *in vitro* cell studies into animal or human models, it is often noticed that responses are not similar in whole body (Lodish et al., 2000). This is because of several factors, few of the important ones being lack of natural chemical environment, physiological clues, and intercellular communication in traditional cell culturing vessels. In addition, a growing trend in drug development is to reduce amount of animal testing for ethical reasons, which gives more incentive to develop better *in vitro* models. It is also the goal of this thesis: to develop new methods for testing of liposomal drug formulations in better *in vitro* conditions which give possibilities to demonstrate benefits of liposomal formulations.

MCMBs are a tool to do cell and organoid experiments in a system that includes more of the chemical environment, flow conditions, stress and intercellular communication within *in vitro* model (Y. Zhao et al., 2019). In this thesis I use commercially available Quasi-Vivo® system to develop model including two cell lines with potential to add further cell lines into same system. Two cell lines used are chosen by their known robustness in culturing conditions, and previous experience in their use. Both cell lines should also be sensitive to chosen test drug DOX on the same range of concentration. According the literature both cell lines are expected to benefit from shear stress, and this system allows to test light activating liposomal formulation and if its off-target toxicity can be reduced. Study is composed of HepG2 viability experiments, combined cell lines viability experiments, determining IC50 value of DOX, and drug formulation testing inside the system (Figure 9).

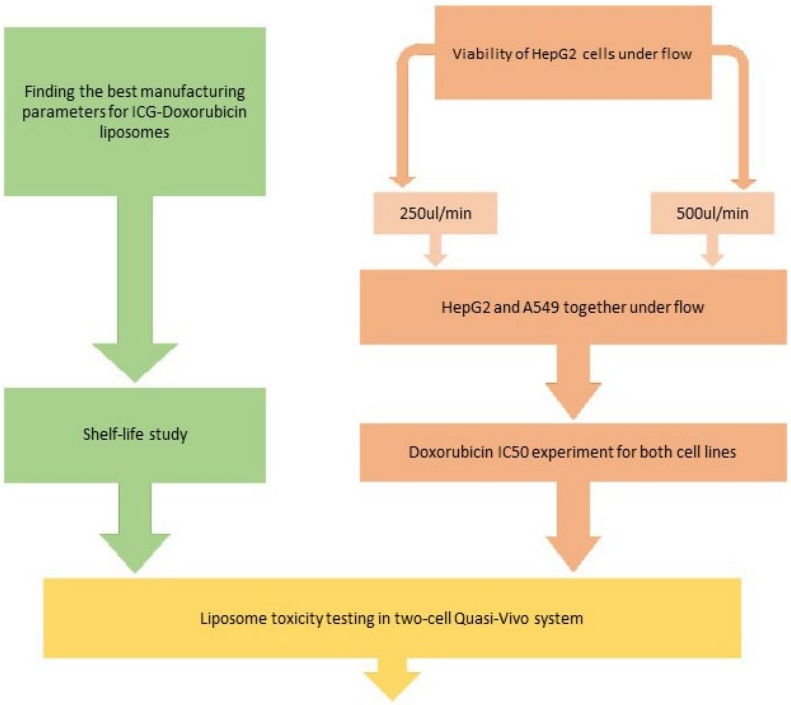


Figure 9: Flowchart to visualize structure of the practical study part of the thesis.

Liposomes are a rather novel method for drug transport, but as such, one with the most well-established manufacturing protocols (Sercombe et al., 2015). Liposomes are also one of only few nanocarriers that have been approved as part of a commercial drug products. Benefits liposomes can give for cancer therapies are prolonged circulation and passively targeted drug exposure, while reducing off-target toxicity (F. Liu & Liu, 1996). Light-activating liposomes are a promising vehicle to better control drug release and further provide us with ways to limit toxicity and long-term side effects (Leung & Romanowski, 2012).

Doxorubicin is rather old but still widely used anthracycline anticancer agent, which toxicity often leads to long lasting impairments, especially related to cardiotoxicity (Thorn et al., 2011). DOX liposomal formulations have already been developed and approved to reduce toxicity, but none of the commercialized products rely on active targeting or controlled drug release. Based on previous experience of manufacturing liposomes in the laboratory, in this thesis I develop a simple and reliable protocol in

making of light-activating ICG-DOX liposomes. Liposomes are prepared by using evaporation-hydration-extrusion-purification protocol, after which active substance and light sensitive dye ICG are incubated in temperature-controlled environment. Determining of suitable incubation times and purification methods will be done by measuring EE of both DOX and ICG and conducting size distribution measurements to ensure liposomes are intact and consistent. For the purposes of *in vitro* experiments, shelf life of ICG-DOX liposomes will be determined related to liposome integrity and ICG disintegration (Figure 9).

This thesis has three main objectives. Firstly, to determine manufacturing parameters to ICG-DOX-liposomes that encapsulation of both DOX and ICG is sufficient, and their shelf-life is long enough for *in vitro* studies. Secondly, confirm that two cell lines can grow in QV system simultaneously in completely shared environment. Thirdly, to test ICG-DOX-liposomes inside this two-cell system and show the possibilities of this system for future use, with for example larger number of cell lines connected.

7. Materials and methods

7.1. Preparation of doxorubicin liposomes

Liposomes were prepared using four lipid components, bases of the protocol were according to previous experiments in the lab and Lajunen et al. (2016). Lipid components were 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (18 Lyso PC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG), all purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). 16.62 mg of phospholipids in molar ratio of 75:15:10:4 (DPPC:DSPC:18 Lyso PC:DSPE-PEG) dissolved in chloroform were pipetted with glass pipettes into glass tube, and mixed.

Evaporation of the chloroform was conducted in rotavapor (Büchi R200, Büchi Labortechnik AG, Flawil, Swizerland) by placing lipid mixture under a nitrogen flow at 470 mbar pressure for 45 minutes and then under 80 mbar for 10 minutes, while submerged in a 63°C water bath and rotating slowly. Successful evaporation would result in uniform lipid film on the bottom of the glass tube. This lipid film was then rehydrated with 500 µl of 240 mM ammonium sulfate solution, pH adjusted to 5.0. Lipids were hydrated for at least 40 minutes in 63°C water bath while vortexing gently and shortly for every 2 minutes to avoid foaming, and after entire lipid film had dissolved, every 5 minutes.

After these steps we would have large variety of sizes of liposomes in ammonium sulfate. Size control of the liposomes was performed with extrusion through a polycarbonate membrane with pore size of 100 nm. Syringe extrusion device and membranes were from Avanti Polar Lipids (Alabaster, AL, USA), and device was heated externally to 63°C allowing lipid bilayers to re-arrange into desired liposome size when

forced through membrane. After liposomes were extruded 11 times through the membrane, they were rapidly cooled externally with cold tap water.

Liposomes were purified from small lipid residues and external phase was changed to HEPES by using size-exclusion gel chromatography. Stationary phase used in the column was dextran polymer Sephandex G-50 (GE Healthcare, Chalfont St. Giles, UK) and mobile phase was HEPES buffered saline of 20 mM HEPES and 140 mM NaCl in pH 7.4. 180 µl of lipid solution was placed into column, and from the end of the column 2 ml fraction was collected. Yield form one liposome batch was total of 4ml. Size distribution of purified liposomes was confirmed with Zetasizer APS dynamic light scattering (DLS) automated plate sampler (Malvern Instruments, Malvern, UK).

Doxorubicin hydrochloride (Merck KGaA, Darmstadt, Germany) was dissolved into HEPES buffered saline or purified water. Liposome solution was divided into four 1000 µl samples and DOX solution was added into mass ratio of 1:20 (DOX:lipids). Liposomes were then incubated in Thermomixer (Eppendorf AG, Hamburg, Germany) in 37°C and with 3000 RPM shaking for 90 minutes. Then ICG dissolved in HEPES buffered saline was added in molar ratio of 1:50 (ICG:lipids) and incubated either for 60 minutes or 180 minutes. After both substances were incubated and incorporated in the liposomes, residues outside the liposomes would be purified with size-exclusion chromatography using either similar column as described before, or disposable PD-10 Sephandex G-25 desalting columns (GE Healthcare, Chalfont St. Giles, UK) to avoid excess sample dilution. Purified liposome solutions were stored in 4°C and protected from light.

Purified liposomes were re-analyzed with DLS to ensure that liposomes had kept their integrity through the incubations, and that purification was successful there being no large particles like precipitated DOX or ICG. DOX content was determined by Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) (Waters, Milford, MA, USA)

equipment, 50 µl of 10% TritonX was added into 250 µl samples before analysis to break down liposome structure. Encapsulation efficiency of ICG was determined by comparing our samples into prepared control liposomes with 1:50 molar ratio of ICG added in lipid film hydration phase. The absorbance of liposome solutions in 800nm was measured using Varioscan Flash plate reader (Waters, Milford, MA, USA). The absorbance was compared between samples and control, and EE was determined.

7.2. Determining shelf-life of Doxoubicin-ICG-liposomes

Shelf-life of prepared liposomes in 4°C was determined regarding the integrity of liposome structure and size, and degradation of ICG. Liposome integrity was determined by measuring liposome size distribution with DLS equipment repeatedly for two-week time. Degradation of ICG was measured repeatedly with absorbance measurements in 800nm with Varioscan® plate reader for similar period of two weeks.

7.3. HepG2 cell viability in QV system

Viability of cell line A549 under flow conditions 250 µl/min and 500 µl/min in QV was determined by previous experiments, so with HepG2 we tested how well this cell line survives in similar flow rates. Our growth media of choice was DMEM with high glucose, supplemented with 10% FBS and 1% PenStrep, components supplied by Merck.

In first viability study we cultivated HepG2 cells in all the QV chambers and compared flow rates of 250 µl/min and 500 µl/min with each other and with no-flow control cultivated on static 24-well plate. Cells were seeded on the first day of the protocol in 24-well plate with density of 150 000 cells/ml and allowed to attach on the surface of round glass coverslips for 48 hours. Meanwhile QV system was sterilized by spraying all the surfaces and tube connections with 70% EtOH and by using the pump to circulate

EtOH inside of the chambers for at least one hour. After this, QV was handled only inside the laminar hood, and EtOH was washed away by circulating 1x DBPS in the system for 48 hours.

On third day, coverslips were carefully transferred into the QV chambers, or into the control well plate. 'Day 0' viability tests were conducted with resazurin AlamarBlue® (Thermo Fisher Scientific, Waltham, MA, USA) assay (AB). 10% resazurin solution in growth media was incubated with the cells for 150 minutes and fluorescence with excitation wavelength of 560nm and emission of 590nm was measured with Varioscan® Flash plate reader. Viability was determined by removing AB background emission from the results. Cells in QV were placed under flow from this point onward (Figure 10).

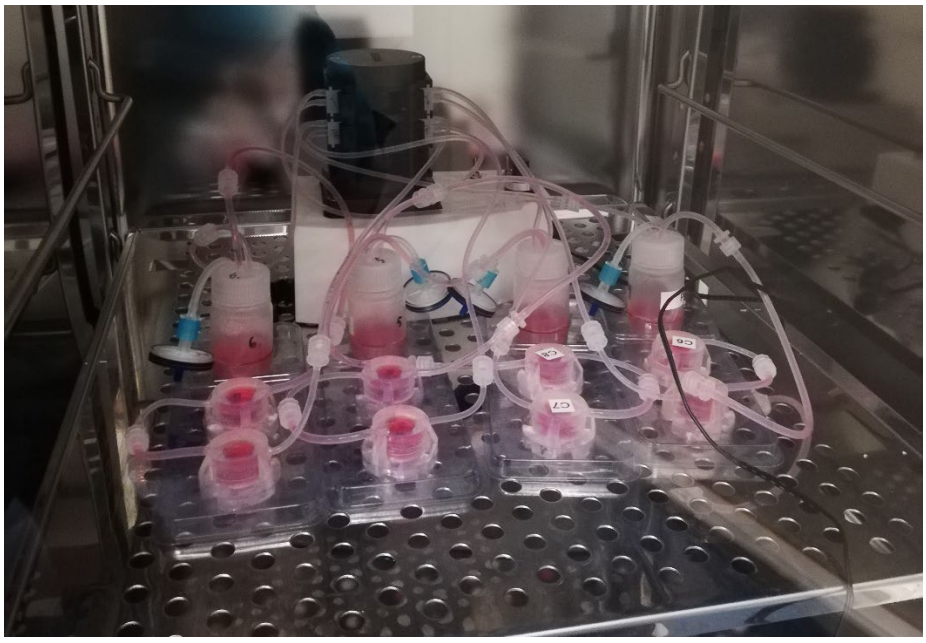


Figure 10: Cell cultivation inside Quasi-Vivo®-system. Peristaltic pump circulates media in the system through two connected cell cultivation chambers. Entire system is placed inside cell cultivation incubator.

Another AB analysis was performed on the 4th day of the experiment protocol, as described before. On the 5th day similar AB analysis was conducted, after which

coverslips were transferred from QV to a 24-well plate and washed twice with 1x DBPS. Then Live/Dead™ Viability/Cytotoxicity Kit for mammalian cells (Thermo Fisher Scientific, Waltham, MA, USA) solution was prepared according to manufacturer's instructions. 500 µl was added on top of the cells and incubated in dark, room temperature conditions for 40 minutes. For cell imaging we used Cytation 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT, USA) with 4 times magnification and Phase Contrast, GFR and RFP filters allowing us to visually separate dead and living cells. Three images per every cell culture were captured and visually analysed Images were captured and further processed with Gen5® software included to microscope system.

After determining how the viability of HepG2 cell line changes depending on the flow rate, we started combining HepG2 and A549 cells in same system. HepG2 cells were seeded in 150 000 cells/ml and A549 in 100 000 cells/ml since it was noted that A549 had significantly faster proliferation. Experimental procedure was similar compared to the described above: QV system consisted of two chambers, one for both cell lines, and four replicant systems were cultivated simultaneously. No-flow controls were cultivated on static well plate.

7.4. IC50 experiment

IC50 values for DOX had to be determined for both cell lines, A549 and HepG2. Cells were seeded in density of 100 000 cells/ml (10 000 cells/well) on a 96-well plate in a formation where cultivated cells are always adjacent to empty wells (Figure 11). This is to prevent possible interference when measuring fluorescence results with plate reader, while using clear well plates. Media used was similar to the one described earlier, HG DMEM with 10% FBS and 1% PenStrep.

Table 2: How test compounds were diluted and placed on the 96-well plate, 1% Triton in 1st column and others respectively. DOX concentration in µM.

1% Triton in DPBS	Water 2% (v/v)	Living control	DOX 0.001	DOX 0.01	DOX 0.1	DOX 1	DOX 10	DOX 50	DOX 100	DOX 200	AB control (no cells)

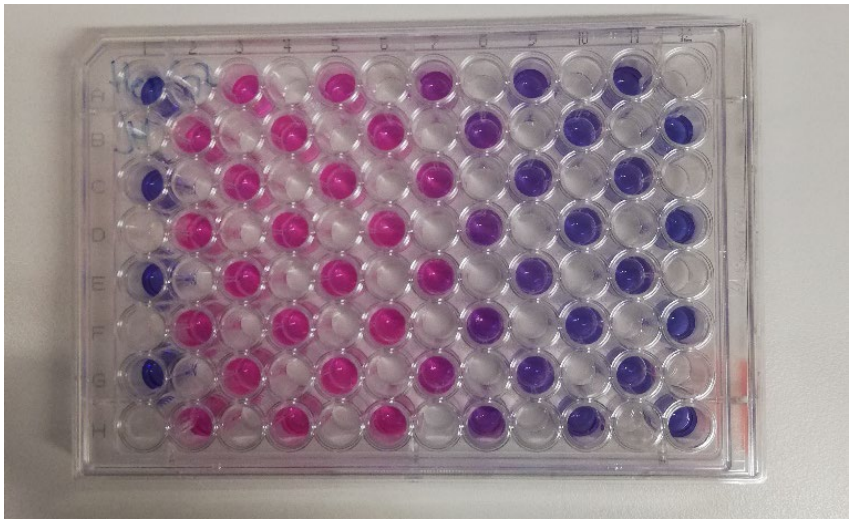


Figure 11: Cells were seeded on the 96-well plate according this composition. They are never adjacent to each other, to avoid possible measurement interference when using clear well-plate. Image is taken after AB incubation.

After seeding the cells, plates were left to incubate for 24 hours to allow cells to attach and proliferate. Then growth media was removed, and test compounds were added, four replicant wells for each concentration were prepared. DOX was first dissolved into pure water in concentration of 10 mM after which it was further diluted with growth media into test concentrations, which were according to table 2. Dead control wells were treated with 1% TritonX, and living control received only growth media.

After plates were incubated 24 hours with test compounds, media was removed and now replaced with 10% AB -reagent in growth media. After incubating plates for two more hours, plates were then placed into Varioscan® flash plate reader and similar

fluorescence analysis was conducted as described earlier. Fluorescence results could then be placed on a curve that was fitted with Prism® statistical analysis and graphing software (GraphPad, San Diego, CA, USA). This protocol was repeated for three times with cells from three different passage numbers, then results were combined and IC50 values were determined.

7.5. Liposome treatment in QV system

Experimental protocol was similar compared to combined cell lines experiment, cells were seeded on the 1st day of the protocol and 'Day0' viability was measured on 3rd day. Then DOX liposomal formulation prepared earlier was added to the systems in drug concentration of 10.4 µM which was close to higher determined IC50 value (10.88 µM for HepG2) to get surely visible effect from the drug. First week of liposome experiments was non-illuminated treatment control.

Next phase was final experiments with liposome treatment and laser illumination. After 'Day0' viability experiments, liposomes were added to the system in DOX concentration of 10.4 µM and incubated with circulation for 3 hours, after which the first chambers in flow direction, seeded with A549 cell line, was illuminated with 808 nm laser with light intensity of approximate 3200 mW/cm^2 and duration of 60 s (Figure 12-A). No-treatment control on a static well plate was also illuminated with similar parameters (Figure 12-C). After these steps, experiment was continued according to the previous protocol including two viability measurements and cell imaging.

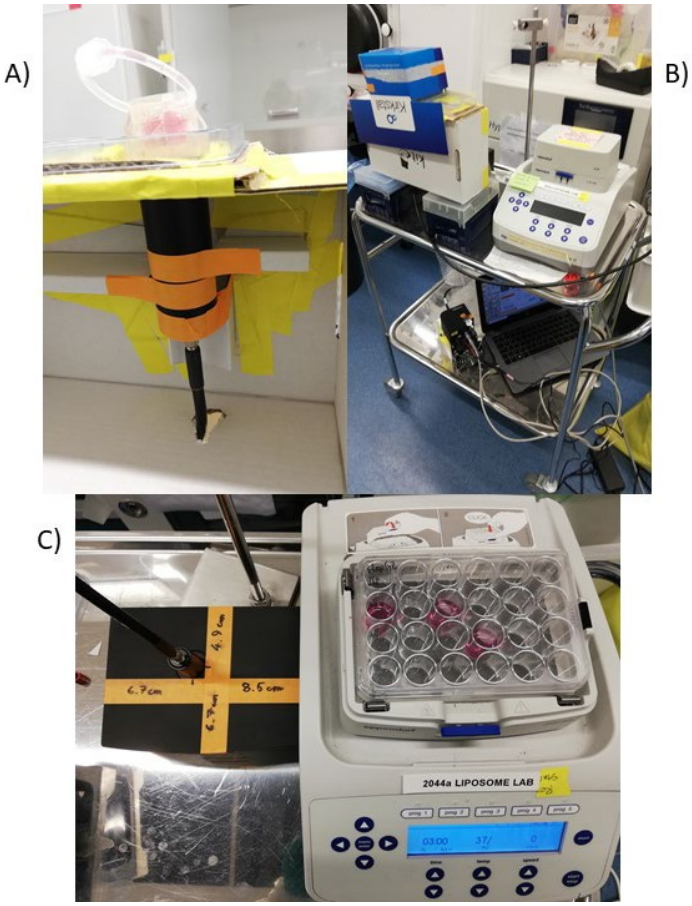


Figure 12: Laser illumination system for cell cultures. A) QV chambers were illuminated from below using cardboard covers and guides. B) Transportable system used to illuminate cell cultures with 808 nm laser. C) Static control was illuminated from above using hood that could be placed tightly upon Thermomixer.

8. Results

8.1. Liposome preparation

First goal of the experiments was to create protocol in manufacturing ICG-DOX-liposomes and determine methods that provide high and reliable encapsulation of IGG and DOX. Preliminary liposome preparation protocol was based on previous experiments in the lab and the work of Lajunen et al. (2016). Different ICG incubation times were tested to achieve the best DOX and ICG encapsulation. Old preliminary protocol used in the beginning of the experiments instructed to dissolve DOX into HEPES, but this led at best <40% EE (Figure 13-A) which would not be sufficient for *in vitro* experiments and led to increased amounts of cytotoxic waste. When DOX was later dissolved in purified water, encapsulation was significantly increased, up to almost 90% (Figure 13-B).

It was already noted in preliminary experiments that when incorporating payload inside liposomes in 37°C, EE of ICG will increase with longer incubation times, but at the same time EE of DOX will decrease. It seemed to be that optimal ICG incubation time lies somewhere between 60-200 minutes. Figure 3 presents how DOX content falls slightly when increasing duration of incubation, but lengthier incubation promotes ICG incorporation (Figure 13-C).

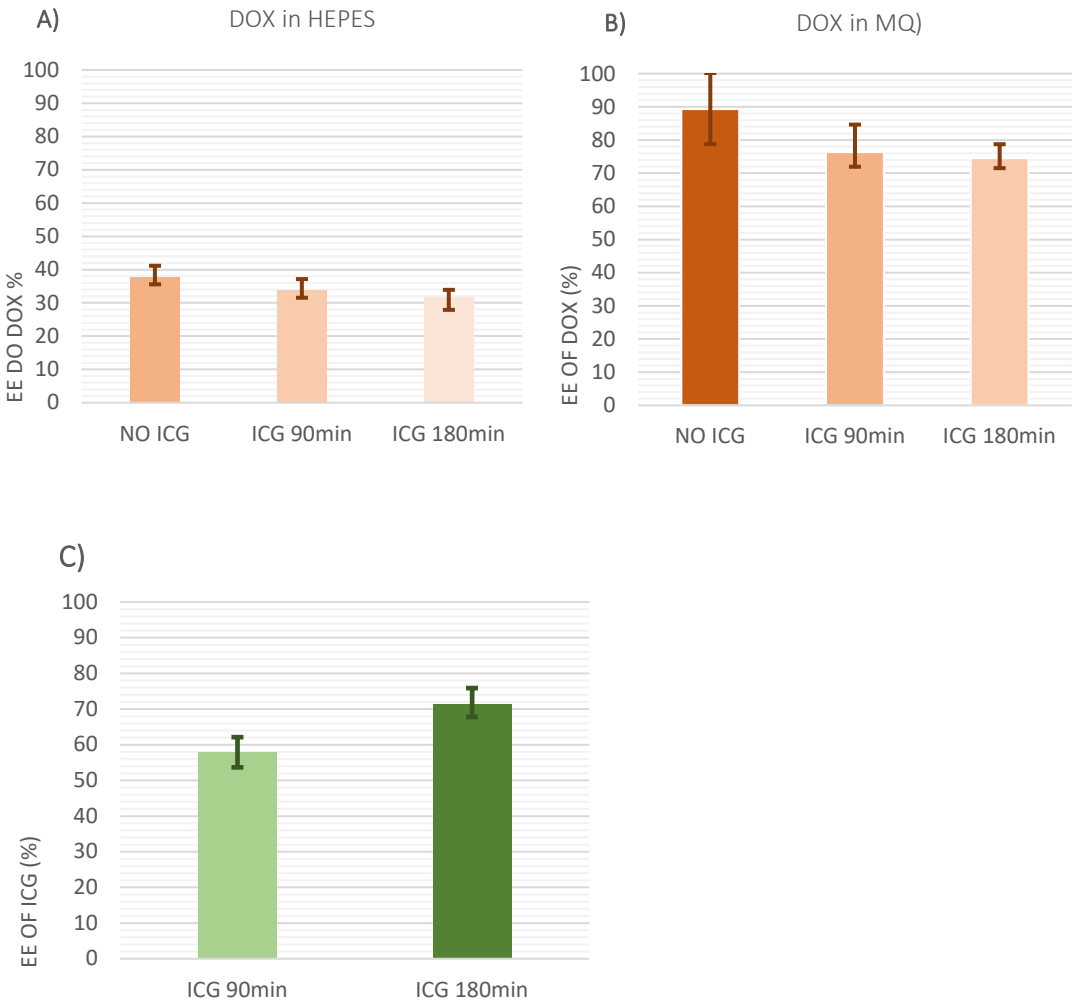


Figure 13: EE of doxorubicin (DOX) when dissolved in HEPES (A) or in MilliQ purified water (MQ) (B). Longer ICG incubation leads to increased drug leakage, but ICG encapsulation on the other hand increases (C). ICG incubated either for 90 or 180 minutes. Results presented as means with +/- SD (n=8 in all groups)

Manufacturing process of liposomes was monitored by determining liposome size with dynamic light scattering measurement device after extrusion and purification and further after DOX and ICG incubations. No significant changes in size distribution were noted after any of the manufacturing stages, average diameter of liposomes after processes was constantly between 120-135 nm which was to be expected with the equipment used. Results of purification with gel extrusion chromatography was monitored also with DLS equipment and described with polydispersity index (PdI).

Samples were determined to be very monodisperse, PdI being in all of the samples less than 0.1, zero meaning sample being completely uniform in size.

8.2. Shelf-life

The shelf-life of DOX-ICG -liposomes was measured regarding to size uniformity and ICG degradation when stored in 4°C refrigerator. According to DLS measurements only small increase in average liposome size of up to 1.5% is noticed (Figure 14) which is not statistically significant change between measurements according to Welch's ANOVA (F (5, 16.051) = 1.827, p = .173) (SPSS® statistics software (IBM®, Armonk, NY, USA)). Also, no change in polydispersity index was observed, which indicates that there is no significant formation of insoluble impurities in the solution. Average PdI stayed < 0.1 for entire test period.

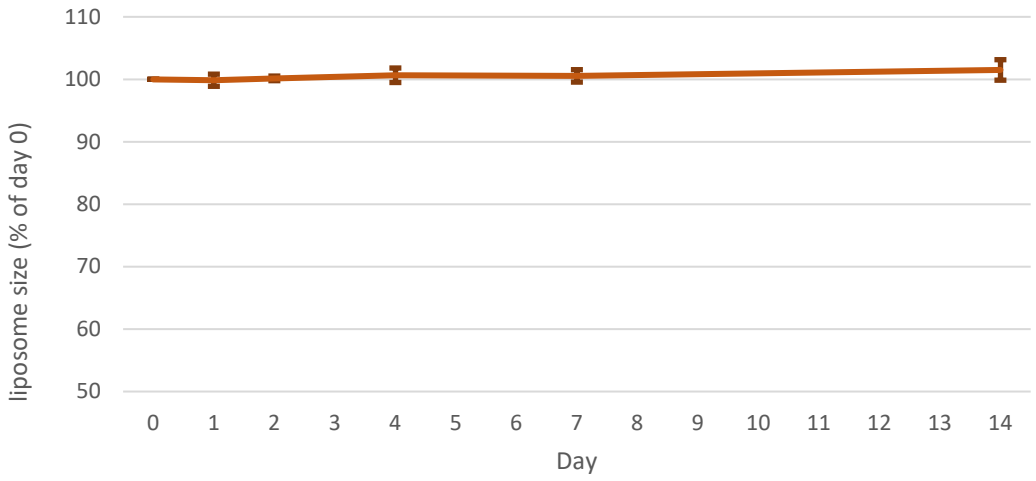


Figure 14: Change in average liposome size during two-week test period. Values presented as mean +/- SD (n=8)

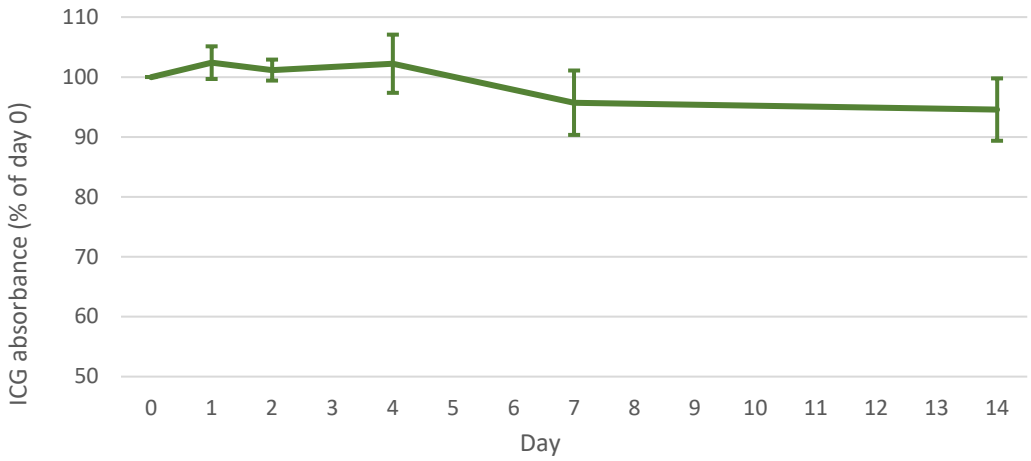


Figure 15: Change in average ICG absorbance during two-week test period. Values presented as mean +/- SD (n=6).

When ICG absorbance was measured for 14 days, decrease during that time was on average 5.5%, and this change between measurements is statistically significant determined by Welch's ANOVA (F (5, 11.799) = 3.592, p = .039) despite increasing variation between samples (Figure 15). Maximal ICG degradation across all samples was less than 12%. Shelf-life of this formulation was determined to be sufficient for our future *in vitro* experiments.

8.3. HepG2 viability under flow

In previous experiments in the lab, it had been established that A549 can survive under both 250 µl/min and 500 µl/min flow conditions. In this thesis the goal is to combine both A549 and HepG2 cell lines under same flow system, so first it was necessary to determine if also HepG2 could survive in QV system. Changes in cell viability during test period was monitored using resazurin assay that measures cell mitochondrial metabolism. HepG2 cell metabolism was measured under flow rates of 250 µl/min and 500 µl/min, and according to the results both flow rates provide at least as high proliferation as static control (Figure 16-A & B), with no significant differences with

repeated measures ANOVA. Figure 16-A presents absolute growth of every cultivation group, but since small differences in seeding and handling of cells can lead to significantly different growth, more informative is to compare subjects with control seeded at the same moment, which is presented at figure 16-B.

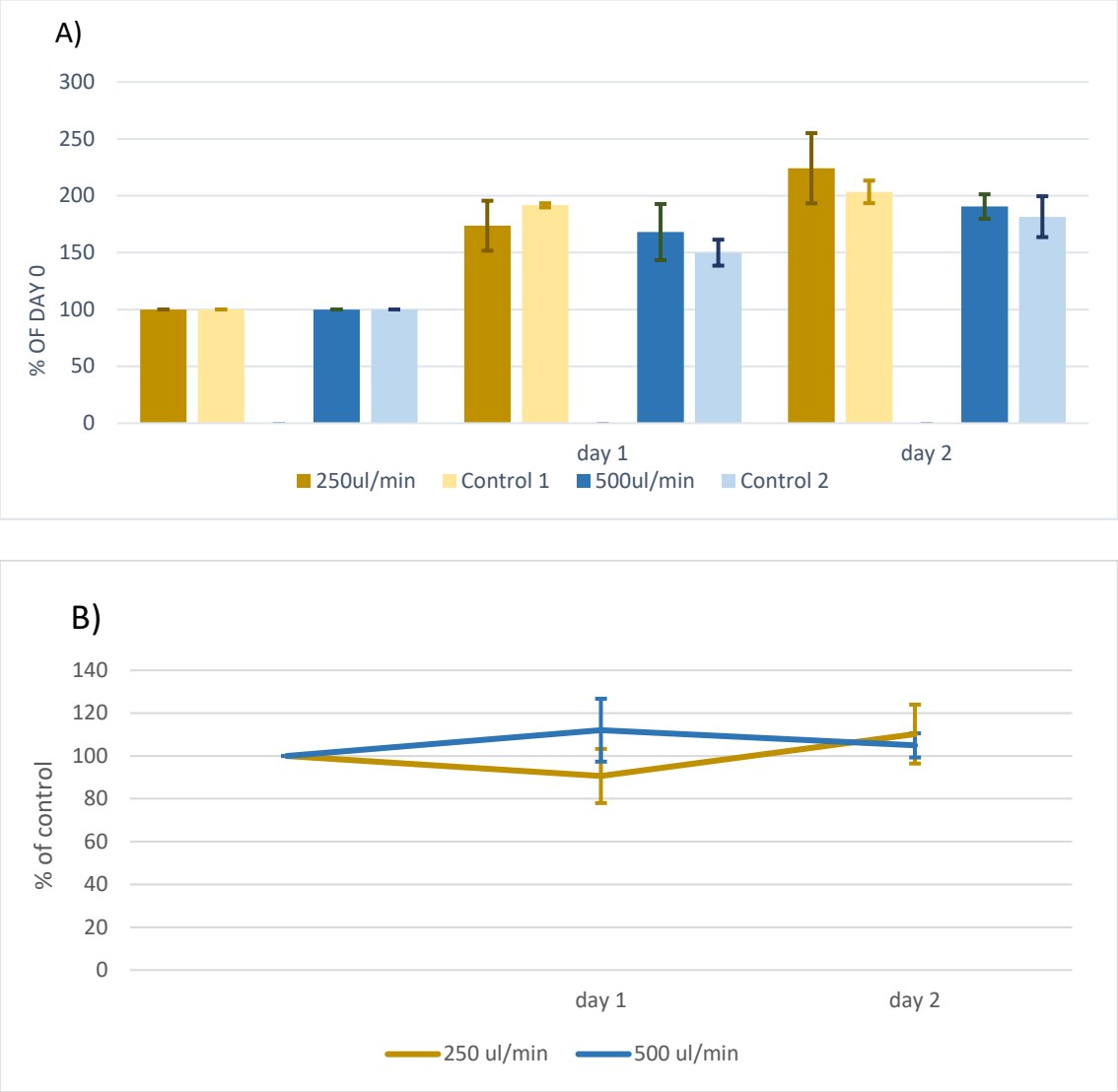


Figure 16: Viability of HepG2 cell line under flow conditions and compared to static control. Table A) presents absolute growth compared to day one, but more relevant is to compare growth with control cells seeded at same time, as presented in table B). This shows that growth in average is slightly faster under both flow conditions, compared to designated controls, and even in the worst case growth is not hindered, no significant differences between groups according to repeated measures ANOVA. Results presented as means +/- SD (n=3 in all groups)

Cells were also visually evaluated after Live/Dead™ kit staining, and no clear differences in morphology could be noted between cells grown under static or flow conditions (Figure 17). All cultures represent HepG2 typical formation of spheroids although this is not as visible in cultures below (Figure 17-C & D) due to cultures reaching higher confluency. No significant number of dead of detached cells are observed, except on some areas near the edges of the glass coverslips, that might have died because of drying. Live/Dead™ kit stains dead cells red and active cells green, when observed through correct filters.

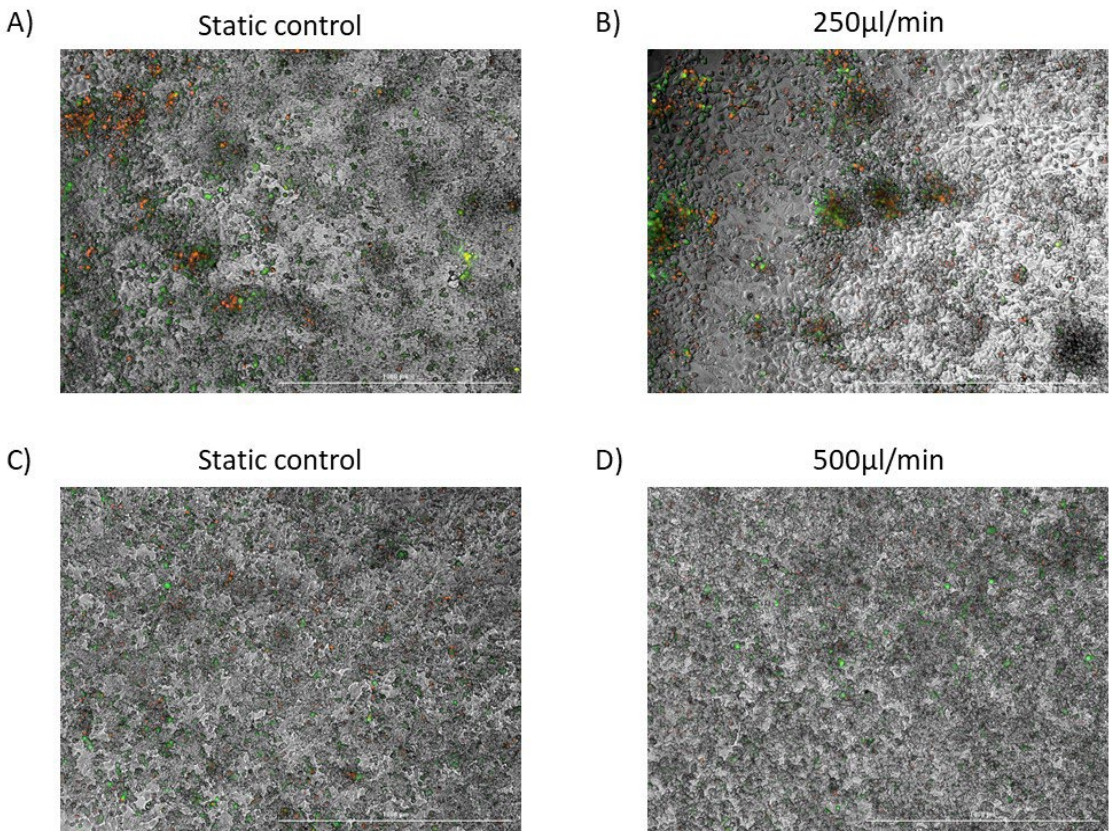


Figure 17: Images captured after Live/Dead™ kit staining from cell cultures under both flow conditions and compared to static. From upper left corner: A) the first week static control, B) 250 µl/min flow culture, C) second week static control and D) 500 µl/min flow culture. No significant differences in morphology are noticed in either flow conditions compared to designated static control. Images captured with Cytation 5 Cell Imaging Multi-Mode Reader and processed with Gen5® software (BioTek Instruments, Winooski, VT, USA).

8.4. Cells in combined flow system

Next step was to investigate how HepG2 and A549 cell lines would grow together in combined cell system under flow conditions, and if there would be significant benefits or disadvantages of cells being in distant contact with each other. Experiments were conducted under 500 µl/min flow. Results are presented in Figure 18 and it shows that both cell lines grow with similar pace when in static conditions or under flow, and they do not hinder the growth of each other. Both cell cultures were also imaged after Live/Dead™ treatment, no significant number of dead cells were observed (Figure 19). Morphology of single A549 cells seemed often slightly more spherical in QV group, this might be a reaction to shear stress, or just results from their handling from QV back to 24 well plate before imaging.

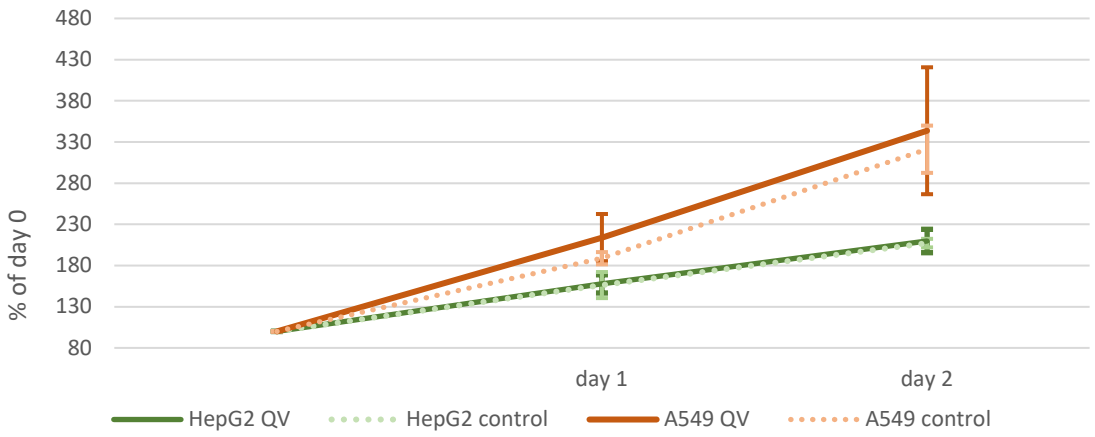


Figure 18: Proliferation of both HepG2 and A549 cell lines in combined system and static controls. Both cell lines grow as fast or faster under flow in combined system than in standard 24 well plate. Absolute growth presented relative to day0 measurements, means and +/- SD (n=3 in all groups). No significant difference between flow and static was observed according to repeated measures ANOVA.

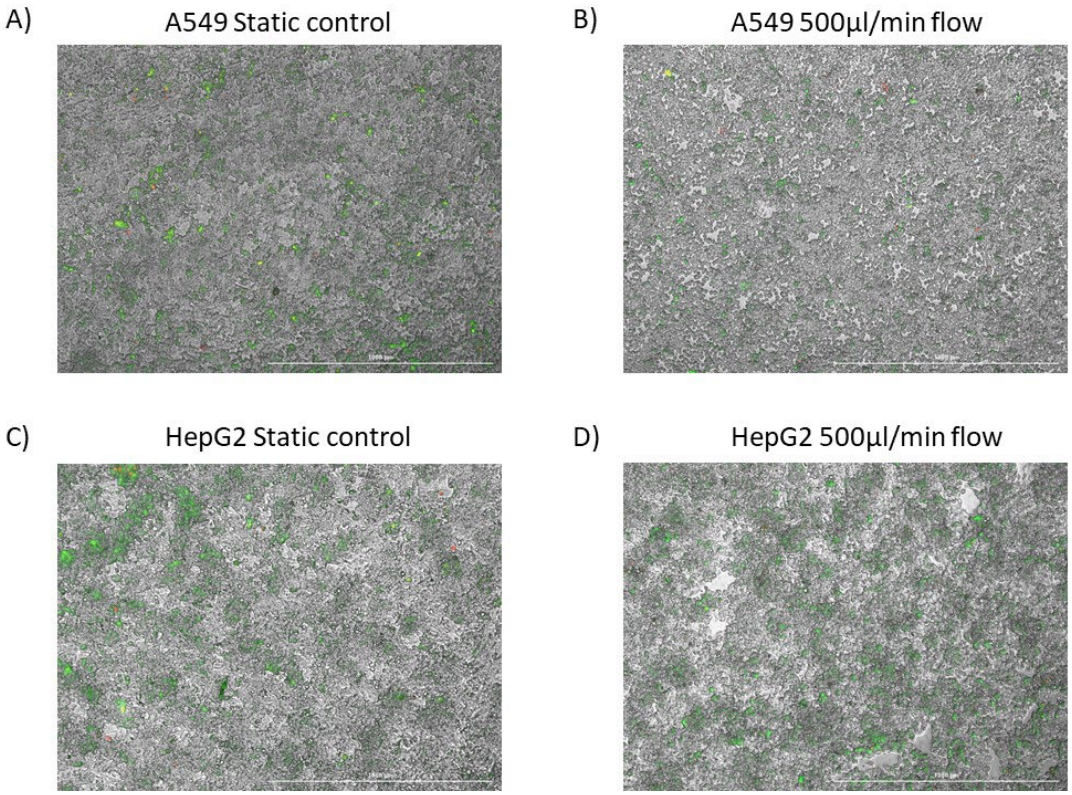


Figure 19: A549 and HepG2 cell lines grown together under 500 µl/min flow (B & D) or separately in static control (A & C). Cells imaged using equipment described previously.

8.5. IC50 experiment

IC50 value was necessary to determine for both cell lines, it would help to estimate the adequate drug dosing for liposome tests. Experiment was conducted three times with cells seeded from three different passage numbers. Acquired results from AB assay were transferred into Prism® statistical analysis software where inhibitory response was fitted into variable slope curve defined by four parameters (Figure 20). Best curve fit provided us with relative IC50 values of 2.88 µM for A549 and 10.88 µM for HepG2 which are in line with relevant literature.

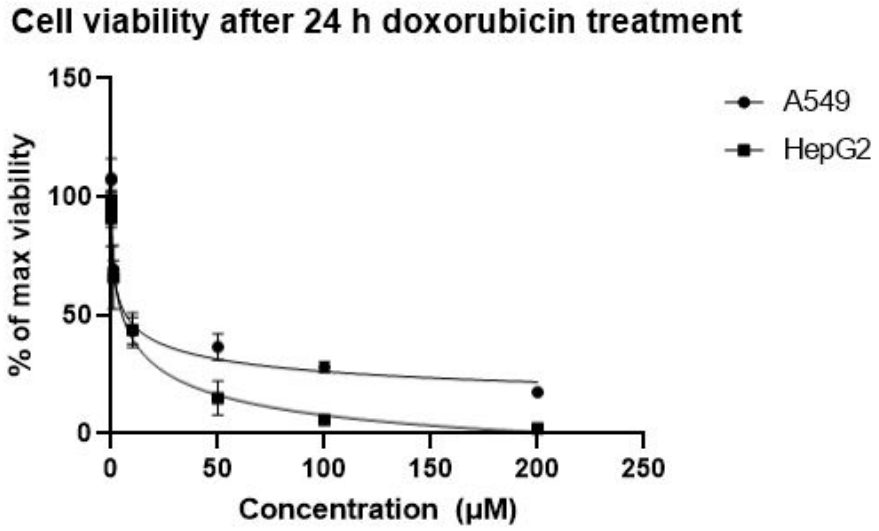
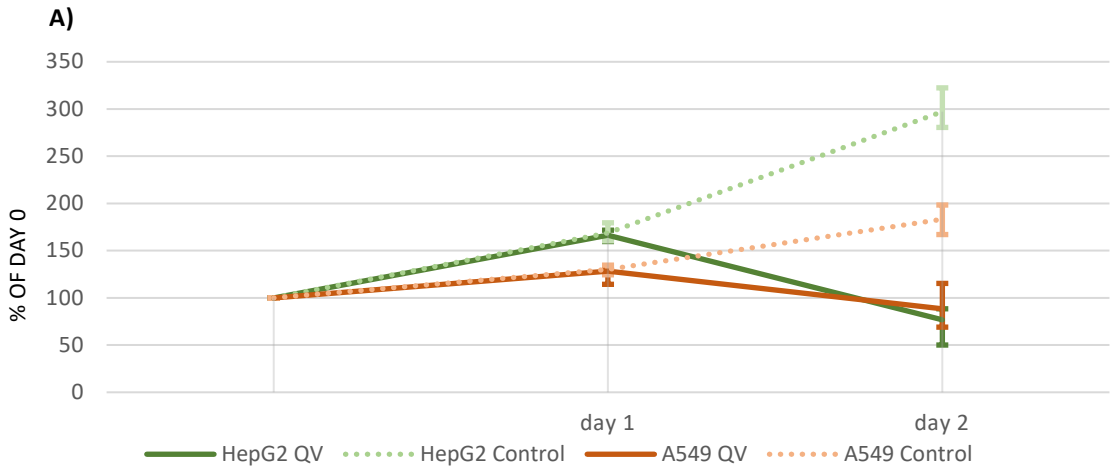


Figure 20: DOX inhibitory effect after 24h incubation. Curve was fitted into variable slope defined by four parameters that provided us the best and the most reliable fit, R^2 values for curves are 0.98 and 0.97. Relative IC50 concentrations are 2.88 µM for A549 and 10.88 µM for HepG2. Figure captured from Prism® statistical analysis software.

8.6. Liposome treatment

In the final phase of the experiment cell lines were treated with ICG-DOX-liposomes after being placed to the QV system. Cells were treated with drug concentration of 10,4 µM and with or without the laser illumination. These preliminary results (Figure 21) show difference in suppression of cell growth when liposome-laser treatment is compared to liposome treatment in dark conditions where control groups are static no-treatment. Non-illuminated treatment groups seem to be able to resist drug effect for the first 24 hours (Figure 21-A), compared to group receiving short illumination that are showing inhibition in day 1 measurements (Figure 21-B & C). Only A549 cell line was illuminated, so drug effect seems to be also transferred through QV system to 'off-target' HepG2 cells. Differences between all treatment groups were significant according to repeated measures ANOVA ($F(2,9) = 319.487$, $p = .001$).


A)
350
300
250
200
150
100
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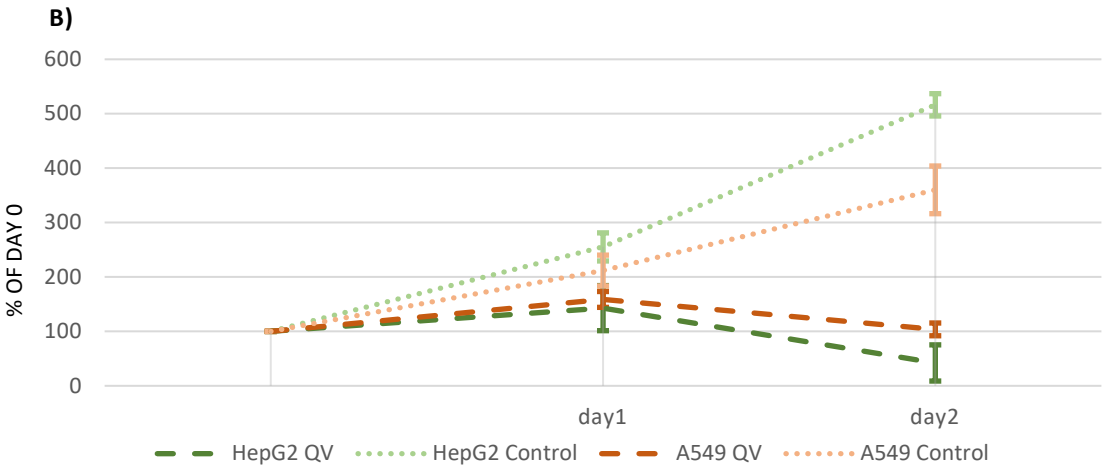
% OF DAY 0
day 1
day 2
HepG2 QV
HepG2 Control
A549 QV
A549 Control


B)
600
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HepG2 QV
HepG2 Control
A549 QV
A549 Control

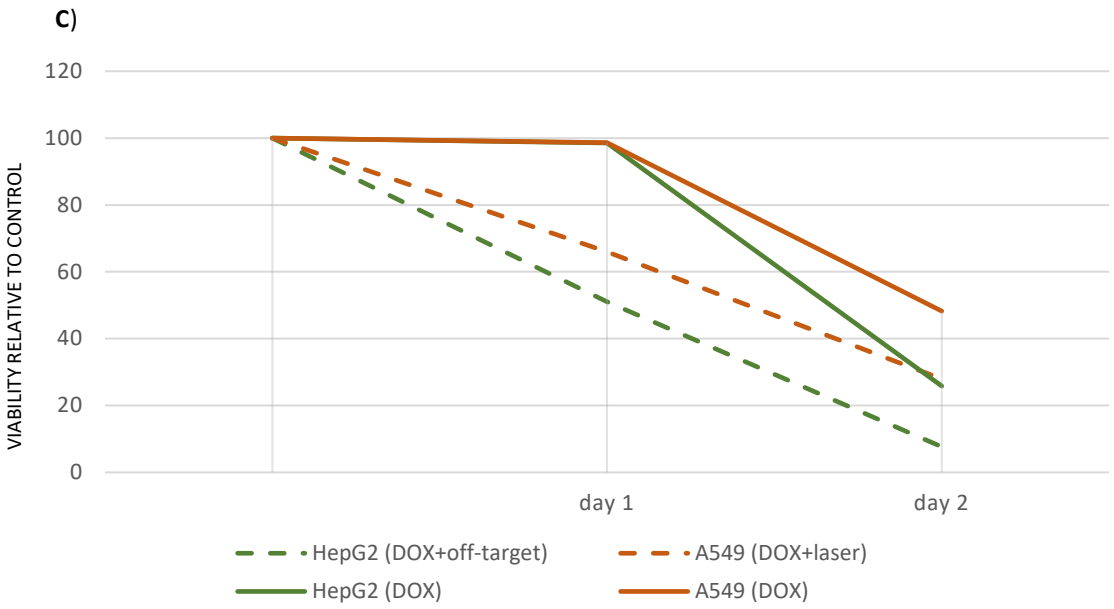

Figure 21: Effect of ICG-DOX-liposome treatment when combined with 808nm laser illumination. A) non-illuminated treatment group, where treated cells continue to proliferate similarly to control for 24 hours, after which drug effect is visible. Control is no-treatment static culture. B) Illumination seems to increase the effectivity of ICG-liposomal formulation, as expected. Control is no-treatment static culture, where A549 cells are illuminated similarly to treatment group. C) Visualisation of the comparison between illuminated and non-illuminated treatment groups. Figures A) and B) presented as means with +/- SD, figure C) presented as means (n=4 with all groups), differences between all treatment groups were significant according to repeated measures ANOVA ($F(2,9) = 319.487$, $p = .001$)

Results of these experiments were also imaged after Live/Dead™ treatment with Cytation5 microscope (Figure 22). Figures 22-A and B present healthy control cells, and when comparing those to non-illuminated treatment cells (22-C, D, E and F) treated groups are showing varying degrees of suppression, where A549 cultures have restricted areas of dead cells, often towards the edge of glass coverslip, while HepG2 cells tend to get affected first when located outside of the cell spheroids. On the laser treatment groups it was more common to find only few A549 cells attached to the glass coverslip (22-G), or when HepG2 cells are really affected by the drug treatment, they often detach and only few residues of cell spheroids are left (22-H).

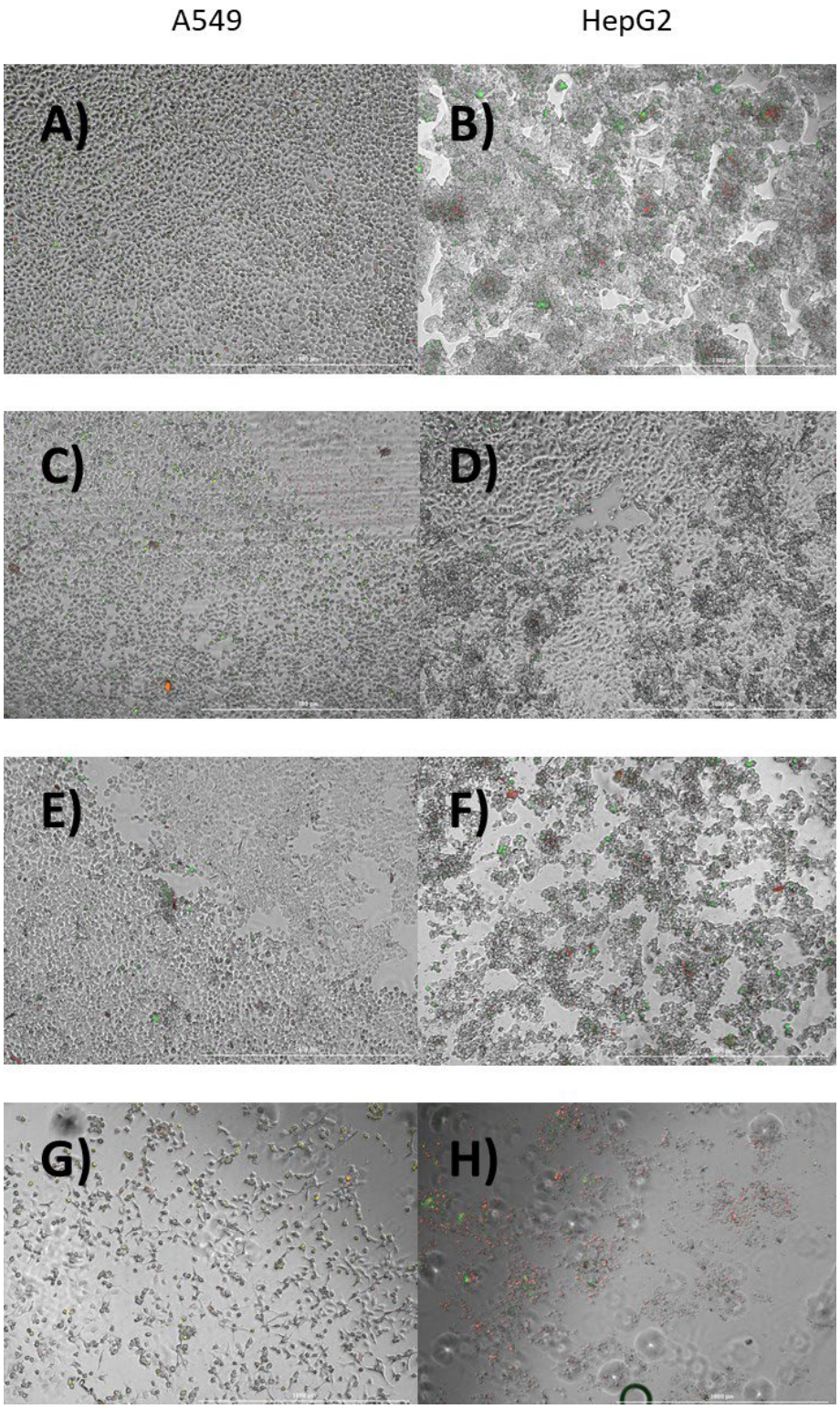

Figure 22: Results of ICG-DOX-liposome treatment. A and B: healthy control cultures, A549 illuminated 60 s with 808 nm laser. C, D, E and F: different degrees of drug effect in non-illuminated treatment groups. G and H: heavily suppressed illuminated treatment cultures. Cells imaged using the same equipment as previously.

9. Discussion

When creating this liposome preparation protocol, the goal was to make liposomes that would be practical to use in further *in vitro* experiments. Although no clear exclusion criteria were determined ahead of the experiments, it was considered according to earlier experiments in the lab that minimum of 70% EE for both DOX and ICG was necessary to achieve. Preliminary protocol first used instructed to dissolve DOX in HEPES, but it was soon noted to lead unacceptably low encapsulation. This is likely to be due to the fact that DOX slowly forms insoluble fibres when in neutral buffers by process of covalent dimerization, further described in article by Yamada (2020). When DOX was in later batches dissolved in purified water, EE improved significantly. ICG encapsulation was sufficient with 180 minutes of incubation, although it should be noted that ICG-powder characteristics change radically if the bottle has been in use for few months. Its solubility seems to decrease by visual inspection and that might also affect its EE which might be relevant to note for future experiments.

Shelf-life of the liposomes was long enough regarding these experiments. Liposome integrity was maintained well for two weeks, according to DLS no aggregation or disintegration was noticed. In the future it would be interesting to find out if there is any leaking of DOX from the liposomes during storage, but at least according to no change in sample PdI there is no formation of DOX aggregates outside the liposomes. ICG degradation was significant but not yet too large to prevent their use.

HepG2 cell line was partly chosen for its known robustness for handling, and it proved to be very capable of proliferating normally inside QV system. It seemed also be able to withstand other handling related to operation of this system, like transferring the glass coverslips with cells on top from the well plate to the QV chamber. Both A549 and HepG2 are possible to incorporate in further combined cell line systems with perhaps larger number of different cell lines in fluid connected chambers. In the future cell

phenotypical changes should be further investigated, for example how the change in A549 morphology affects cell behaviour, and how QV environment changes HepG2 enzymatic activity, if at all. Flow rate of 500 µl/min was chosen for these experiments basically just to make the effect of the flow conditions more significant, since difference in viability was not observed. In future phenotypical experiments it would naturally be important to do more in-depth comparative analysis of different flow rates.

QV system is according to lab experience functional and modifiable environment for cell cultivation and drug testing, although some maintenance issues related to pump tubing led a few times inadequate circulation of the media and loss of the samples, which then lengthens the time needed for experiments. Transferring coverslips with pincers was in addition a rather error prone process, and many of the cell cultures were damaged to the point of them being no good for use, so when seeding the cells, it was necessary to always seed at least double the number of glass coverslips needed for the experiments. Also, when on the last day of the protocol coverslips were recovered from the QV system to the 24-well plate for imaging, samples were sometimes destroyed, which meant that it was not possible to image all the cell cultures. With more experience and training it would be possible to reduce the loss of samples, but it might also be beneficial to develop more methods to transport cell cultures into the system and out of it. Illumination of QV chambers was rather simple using cardboard stands made for purpose, and with no unbearable breach to the system sterility. In the future developing of some frame for the laser that would be easier to sterilize, would be necessary for lab work, at least if parts are in direct contact with cell culture vessels.

Liposomal experiments in QV depict that light-activation of ICG-DOX-liposomes makes visible difference in drug efficacy. Both cell lines seem to be able to resist doxorubicin effect for the first 24 hours if the cultures are not illuminated. When illuminated, suppression of growth in both cell chambers is noticeable already after first day. Only

A549 cells are illuminated, so the drug effect seems to be spreading through the QV tubing. This can be affected by number of factors.

DOX is very water-soluble molecule, so although fluid circulation was not on during the illumination, it was resumed instantly after so released doxorubicin could have been transferred along media to the HepG2 cells. With hydrophobic drug molecule this might not have been the case, or with longer waiting period after illumination. Administered drug dose 10.4 µM was close to the higher determined IC50 value (10.88 µM for HepG2), and this was in fact slightly higher than what was first intended due to small error with calculations. If drug dosage would have been lower, closer to the IC50 of A549 (2.88 µM), this might have also proven to be effective in suppressing A549 but without similar off-target effects. Due to technical issues with peristaltic pump and death of some cell cultures, liposome experiments were not repeated for second week. When comparing illuminated group to non-illuminated control, although differences between groups were statistically significant and results were constant between samples, but since handling of cells can have large effect on the results it would be good to confirm these results by repeating these experiments again in the future, to further reduce the chance that human errors might have contributed these results.

10. Conclusion

This thesis was investigating possibilities of using QV-based multi-cellular system in light-activating liposome efficacy and toxicity testing. DOX-ICG-liposomes were successfully and repeatedly manufactured with encapsulation of both molecules greater than 70%, and their shelf-life was determined to be sufficient for *in vitro* testing. HepG2 cell line survives in QV system under flow and combining two cell lines via shared media can be done with no loss in viability. When testing DOX-ICG-liposomes in two cell system, there is significant difference between illumination and dark treatment, but to confirm these results repetition is needed. Off-target effect of liposome disintegration and drug release seems to be transferred through QV system, at least if drug is water-soluble and dose large enough.

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